1-1-2013

Epigenomic and Transcriptional Regulation of Hepatic Metabolism by REV-ERB and Hdac3

Dan Feng
University of Pennsylvania, jilldfeng@gmail.com

Epigenomic and Transcriptional Regulation of Hepatic Metabolism by REV-ERB and Hdac3

Abstract

Metabolic activities are regulated by the circadian clock, and disruption of the clock exacerbates metabolic diseases including obesity and diabetes. Transcriptomic studies in metabolic organs suggested that the circadian clock drives the circadian expression of important metabolic genes. Here we show that histone deacetylase 3 (HDAC3) is recruited to the mouse liver genome in a circadian manner. Histone acetylation is inversely related to HDAC3 binding, and this rhythm is lost when HDAC3 is absent. Diurnal recruitment of HDAC3 corresponds to the expression pattern of REV-ERBα, an important component of the circadian clock. REV-ERBα colocalizes with HDAC3 near genes regulating lipid metabolism, and deletion of HDAC3 or Rev-erbα in mouse liver causes hepatic steatosis. Thus, genomic recruitment of HDAC3 by REV-ERBα directs a circadian rhythm of histone acetylation and gene expression required for normal hepatic lipid homeostasis. In addition, we reported that the REV-ERBα paralog, REV-ERBβ also displays circadian binding similar to that of REV-ERBα. REV-ERBβ also recruits HDAC3 and protects the circadian clock and hepatic lipid homeostasis in the absence of REV-ERBα. REV-ERBs are indeed essential components of the circadian clock. Furthermore, we discovered that REV-ERBs competes with RORα for genomic binding at Bmal1 and Npas2 genes, and drives a diurnal binding of RORα. We then identified thousands of competing sites by RORα ChIP-seq, many of which are in close proximity of clock and metabolic genes. We also discovered many RORα binding sites with no rhythmic RORα binding or rhythmic RORα binding in-phase with REV-ERBs. Collectively, these findings indicate that REV-ERBs, HDAC3 and potentially RORα mediate the epigenomic and transcriptional regulation of liver metabolism by the circadian clock.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Mitchell A. Lazar

Keywords
Circadian Rhythms, Epigenomic, HDAC3, Liver Metabolism, REV-ERB, Transcription

Subject Categories
Genetics | Molecular Biology

EPIGENOMIC AND TRANSCRIPTIONAL REGULATION OF HEPATIC METABOLISM BY REV-ERB AND HDAC3

Dan Feng

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania
in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

2013

Supervisor of Dissertation

*Signature*_____

Mitchell A. Lazar, M.D., Ph.D.
Sylvan H. Eisman Professor of Medicine and Genetics

Graduate Group Chairperson

*Signature*_____

Daniel S. Kessler, Ph.D.
Associate Professor of Cell and Developmental Biologymkm

Dissertation Committee:
Klaus H. Kaestner, Ph.D., Thomas and Evelyn Suor Butterworth Professor in Genetics
Gerd A. Blobel, M.D., Ph.D., Professor of Pediatrics
Patrick Seale, Ph.D., Assistant Professor of Cell and Developmental Biology
Amita Sehgal, Ph.D., John Herr Musser Professor of Neuroscience

To my parents, Yuanhua Wang and Qingyan Feng

Acknowledgements

First and foremost, I would like to thank my mentor Mitch for his guidance and support. His advice and encouragement have helped me developed as an independent scientist, and his passion for science and medicine has motivated me to pursue my own career in biomedical research and medicine. I am also very grateful to work in the vibrant and collaborative lab he has created. I would like to thank many members of the Lazar lab, past and present, for their help and friendship.

I want to express my gratitude to my thesis committee: Klaus H. Kaestner who served as chair, Gerd A. Blobel, Amita Sehgal and Patrick Seale for their advice, constructive criticism and time.

I would like to acknowledge the Cancer Biology program in the Cell and Molecular Biology graduate group. I particularly want to thank M. Celeste Simon, Brian D. Keith and Andrei Thomas-Tikhonenko for their advice and support. The administrators of the Cell and Molecular Biology graduate group have also been very supportive and helpful.

I am truly thankful and appreciative of all the people who helped me with the work presented in this dissertation. Specifically, Martina I. Lefterova provided invaluable advice and experimental guidance when I first joined the Lazar lab and started my projects. Zheng Sun and I collaborated to study the function of HDAC3 in liver, and Anne Bugge worked closely with me on Rev-erbα and Rev-erbβ. Tao Liu, X. Shirley Liu, Bin Fang performed the bioinformatics analyses. David J. Steger helped me enormously

ABSTRACT

EPIGENOMIC AND TRANSCRIPTIONAL REGULATION OF

HEPATIC METABOLISM BY REV-ERB AND HDAC3

Dan Feng

Mitchell A. Lazar

Metabolic activities are regulated by the circadian clock, and disruption of the clock exacerbates metabolic diseases including obesity and diabetes. Transcriptomic studies in metabolic organs suggested that the circadian clock drives the circadian expression of important metabolic genes. Here we show that histone deacetylase 3 (HDAC3) is recruited to the mouse liver genome in a circadian manner. Histone acetylation is inversely related to HDAC3 binding, and this rhythm is lost when HDAC3 is absent. Diurnal recruitment of HDAC3 corresponds to the expression pattern of REV-ERBα, an important component of the circadian clock. REV-ERBα colocalizes with HDAC3 near genes regulating lipid metabolism, and deletion of HDAC3 or Rev-erbα in mouse liver causes hepatic steatosis. Thus, genomic recruitment of HDAC3 by REV-ERBα directs a circadian rhythm of histone acetylation and gene expression required for normal hepatic lipid homeostasis. In addition, we reported that the REV-ERBα paralog, REV-ERBβ also displays circadian binding similar to that of REV-ERBα. REV-ERBβ also recruits HDAC3 and protects the circadian clock and hepatic lipid homeostasis in the absence of REV-ERBα. REV-ERBs are indeed essential components of the circadian clock. Furthermore, we discovered that REV-ERBs competes with RORα for genomic binding

at Bmal1 and Npas2 genes, and drives a diurnal binding of RORα. We then identified

thousands of competing sites by RORα ChIP-seq, many of which are in close proximity

of clock and metabolic genes. We also discovered many RORα binding sites with no

rhythmic RORα binding or rhythmic RORα binding in-phase with REV-ERBs.

Collectively, these findings indicate that REV-ERBs, HDAC3 and potentially RORα

mediate the epigenomic and transcriptional regulation of liver metabolism by the

circadian clock.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

Chapter 1

General Introduction

1.1 Circadian Regulation of Metabolism

The approximately 24 hour cycling of light and dark drives the cyclic changes in the living environments for most organisms on Earth, from cyanobacteria to human beings. To adapt to changing environments, organisms anticipate the periodic changes and adjust their activities with the time of the day using an internal 24 hour clock system, known as the circadian clock. In human beings and other mammals, the clock governs many important behaviors and physiological processes including sleep/wake, feeding, body temperature, hormone secretion and metabolism.

Human beings are diurnal creatures. We conduct most of our activities during the day, including feeding, exercising and working, and rest at night. Circadian clocks in our bodies provide time cues for activities, and meanwhile synchronize the metabolic reactions with the anticipated activity cycles. The synchronization of behaviors and metabolism by the clock ensures the energy supply and maintains the internal homeostasis. However, this delicate system has been increasingly challenged in modern society. Modern life is characterized by increase in night activities, for instance, shift work, overtime work, night eating, sleep disruption and deprivation. Misalignment of activities with the internal clock and metabolic rhythms could disrupt the clock and energy homeostasis. Evidence suggests that shift workers have a higher risk of metabolic diseases, including obesity, diabetes, metabolic syndromes and cardiovascular diseases (Antunes et al., 2010; Wang et al., 2011). Similar effects were also observed with sleep deprivation, sleep disruption and night eating (Reviewed in Huang et al., 2011). In

recognition of these concerns, much recent research focuses on the crosstalk between the circadian clock and metabolism.

1.1a The Molecular Clock System

The cell-autonomous clock machinery consists of several transcriptional-translational feedback loops, which allows autonomous oscillation with a period of approximately 24 hours (Figure 1.1). In mammals, the first feedback loop in the basic clock machinery contains a heterodimer of transcription activators the brain and muscle ARNTL-like protein 1 (BMAL1) and the circadian locomoter output cycles kaput (CLOCK). BMAL1/CLOCK activates transcription of Crytochromes (CRYs) and Periods (PERs). CRY and PER proteins negatively regulates their own expression by binding BMAL1/CLOCK and inhibiting their transcriptional activity (reviewed in Levi and Schibler, 2007). Another critical feedback loop drives the cyclic transcription of BMAL1 using nuclear receptor (NR) REV-ERBα/REV-ERBβ. BMAL1 activates Rev-erbα transcription, which then suppresses Bmal1 transcription (Preitner et al., 2002). The clock machinery is featured by the redundancy of their components: BMAL1/BMAL2, CLOCK/NPAS2 (neuronal PAS domain containing protein 2), CRY1/CRY2, PER1/PER2/PER3, REV-ERBα/REV-ERBβ (DeBruyne et al., 2007; van der Horst et al., 1999; Shi et al., 2010; Zheng et al., 2001) (Figure 1.1). Other factors that are important for clock function include the Retinoid-related orphan receptor RORs (RORα, RORβ, RORγ), which activate transcription of BMAL1 and REV-ERBα, and are repressed by REV-ERBα (reviewed in Jetten, 2009). Levels of the clock proteins are also subject to posttranslational regulation by casein kinases (CKIε and CKIδ), and ubiquitin E3 ligase

β-TrCP, FBXL3 and ARF-BP1/PAM (Yin et al., 2010; reviewed in Gallego and Virshup, 2007; Levi and Schibler, 2007).

Although the transcriptional-translational clocks are ubiquitous in mammalian cells, non-transcriptional mechanisms are aslo sufficient to sustain circadian oscillation. In pico-eukaryotic alga *Ostreococcus tauri*, oxidation of peroxiredoxin proteins undergoes circadian cycles independent of its transcriptional circadian clock (O'Neill et al., 2011). Recently this mechanism was shown to be conserved in higher organisms including human, at least in human red blood cells (O'Neill and Reddy, 2011). The wide-spread presence of transcription-independent redox oscillations suggests that crosstalk between metabolic cycles and circadian clocks has long played a significant role in both physiology and evolution.

In mammals, the basic clock machinery is present in most organs, assembled in a hierarchical system in which the central clock can entrain peripheral clocks (Figure 1.2). The central clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, while peripheral clocks are present in most organs, including metabolic organs such as liver, adipose, heart, muscle and kidney (reviewed in Dibner et al., 2010; Welsh et al., 2010). Both the central clock and peripheral clocks can sense and respond to environmental changes, and keep synchronized with the environment. The environmental cues that can rest the clock are known as Zeitgebers ("time-giver"). The predominant Zeitgeber for the central clock is light, which is sensed by retina and signals directly to SCN. The central clock can then entrain the peripheral clocks through neuronal and

hormonal signals, as well as body temperature, aligning all clocks with the external light/dark cycle (Balsalobre et al., 2000; Brown et al., 2002; reviewed in Dibner et al., 2010; Welsh et al., 2010). Peripheral clocks in metabolic tissues are also entrained by the central clock through feeding/fasting cycles (Figure 1.2). Fasting/feeding alters the levels of key endocrine hormones and the intracellular metabolic state, which can modulate the function of peripheral clocks. When restricted food availability shifts the fasting/feeding cycles, peripheral clocks can be reset separately from the central clock, suggesting that food availability is the predominant Zeitgeber for these clocks (Damiola et al., 2000; Stokkan et al., 2001).

1.1b Circadian Oscillation of Metabolism

Metabolic activities undergo diurnal changes, as reflected by the oscillating hormone levels in blood and rhythmic activities of metabolic pathways. Hormones mediate the crosstalk between the central nervous system and major metabolic organs, and are essential to metabolic homeostasis. Some of them are secreted in a circadian manner, including insulin and glucagon from pancreas, adiponectin and leptin from adipose, and ghrelin from stomach (reviewed in Froy, 2007). Many metabolic pathways display rhythmic activities, consistent with levels and activities of the rate-limiting enzymes. For instance, in liver, gluconeogenesis peaks in the daytime with PEPCK activity (Kida et al., 1980), and cholesterol and bile acid synthesis peaks around midnight, same as HMG-CoA reductase (HMGCR) activity (Back et al., 1969; Higgins et al., 1971). Rhythmic activities were also observed in xenobiotic metabolism, glycogen metabolism and amino

acid metabolism, and in other metabolic tissues, such as fat (reviewed in Davidson et al., 2004; Gimble and Floyd, 2009).

Transcriptomic studies in different metabolic organs and SCN revealed that a large proportion of the whole transcriptomes, from 3% to 20%, undergo circadian oscillation (Akhtar et al., 2002; Balsalobre et al., 1998; McCarthy et al., 2007; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Zvonic et al., 2006). The circadian transcriptomes are tissue-specific and tightly correlated with cellular functions, supporting that circadian clocks control metabolic activities through transcriptional regulation of key metabolic genes. In liver, it includes genes involved in glucose, lipid and xenobiotic metabolism; In skeletal muscle, it mostly contains genes involved in lipid metabolism; In the SCN, it includes genes involved in protein-neuropeptide synthesis, secretion and degradation and regulation of mouse locomoter activity (Akhtar et al., 2002; McCarthy et al., 2007; Panda et al., 2002). while Circadian control of activities can also be mediated through translational and post-translational regulation (Reddy et al., 2006).

1.1c The Molecular Clock Controls Metabolic Activities

The role of the circadian clock in metabolic regulation is well supported by genetic evidence that mutation in clock genes disturbs rhythmic expression of key metabolic genes and causes metabolic disorders. Key metabolic pathways under clock control will be discussed in this section. In addition, other metabolic or related pathways are also regulated by the circadian clock, including cardiovascular function and inflammation (reviewed in Bass and Takahashi, 2010).

Glucose homeostasis Loss of BMAL1 attenuates the diurnal variation in glucose and triglyceride levels, impairs gluconeogenesis and shows glucose intolerance, while the Clock mutant mice (ClockΔ19) develop hyperglycemia and hypoinsulinemia (Lamia et al., 2008; Rudic et al., 2004; Turek et al., 2005). Both genetic models suggest that BMAL1/CLOCK plays a critical role in glucose homeostasis. The study was followed by tissue-specific deletion of Bmal1 in liver and pancreatic β cells, both of which support the critical role of peripheral clocks in metabolic tissues. Loss of BMAL1 in liver abolishes rhythmic expression of glucose metabolic genes, e.g. Pepck and Glut2, and leads to hypoglycemia only in the fasting phase of the day, while loss of BMAL1 in pancreatic β cells impairs insulin secretion (Lamia et al., 2008; Marcheva et al., 2010).

Other clock components also play a role. CRYs inhibit gluconeogenic gene expression, probably through regulating CREB activity. Therefore, hepatic depletion of CRY1/2 increases circulating glucose, while CRY1 overexpression reduces fasting blood glucose and improves whole body insulin sensitivity in *db/db* mice (Zhang et al., 2010). CRYs also transrepress glucocorticoid-induced Pepck transcription, and loss of CRYs results in glucose intolerance (Lamia et al., 2011). Similarly depletion of both REV-ERBs increases fasting blood glucose (Cho et al., 2012). And RORα is known to activate G6pase through SRC-2 and BAF60a (Chopra et al., 2008; Tao et al., 2011).

Lipid metabolism Clock mutation and Bmal1 deficiency also impairs lipid metabolism, as shown by hyperleptinemia, hyperlipidemia and hepatic steatosis (Shimba et al., 2011;

Turek et al., 2005). The mechanism behind this remains unclear. REV-ERB agonists inhibit lipid and cholesterol synthesis in liver and fat, and promote fatty acid and glucose oxidation in muscle, resulting in increased energy expenditure. These agonists significantly improve dyslipidemia and hyperglycaemia in a diet-induced obesity model (Solt et al., 2012). In addition, BMAL1 and REV-ERBα both regulates adipocyte differentiation (Laitinen et al., 2005; Shimba et al., 2005; Wang and Lazar, 2008).

Bile acid metabolism REV-ERBα is an important regulator of bile acid synthesis. Genetic ablation of REV-ERBα lowers bile acid synthesis and decreases bile acid accumulation in the liver, correlated with altered phase and total decrease of Cyp7a1 expression (Duez et al., 2008; Le Martelot et al., 2009). REV-ERBα may indirectly activates Cyp7a1 via E4bp4 and SHP, or via LXR (Duez et al., 2008; Le Martelot et al., 2009). REV-ERBα also regulates SREBP function through its regulator INSIG2 and thus the SREBP targets involved in cholesterol and lipid metabolism (Le Martelot et al., 2009). Loss of BMAL1 increases circulating cholesterol, and loss of PER1/2 up-regulates bile acid synthesis and results in hepatic cholestasis (Ma et al., 2009; Shimba et al., 2011).

1.2 Clock and Epigenome

1.2a Transcriptional Regulation by the Circadian Clock

How does the circadian clock drive rhythmic gene expression in different tissues? In the core clock machinery, BMAL1/CLOCK, REV-ERBs, PERs, CRYs and RORs are all transcription regulators. BMAL1/CLOCK displays rhythmic genome binding and can drive the rhythmic expression of its targets (Rey et al., 2011; Ripperger and Schibler,

2006). REV-ERBα directly regulates gluconeogenic enzymes G6pase and PEPCK (Yin et al., 2007). More importantly, the core clock machinery can drive circadian expression of many transcription factors, thus extending and enhancing its regulatory function. Among these factors are nuclear receptors (NRs), such as retinoic acid receptors (RARs), TRs, peroxisome proliferator-activated receptors (PPARs), GR and short heterodimer partner (SHP) (Yang et al., 2006). Other transcription factors include the three members of the Prolin Acidic Amino acid Rich (PAR) domain basic leucine zipper proteins albumin D-site binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatocyte leukemia factor (HLF), and the related protein E4BP4. All four of these proteins bind to D-boxes, but while DBP, TEF, and HLF are activators, E4BP4 is a repressor. Many of these factors are direct targets of the clock. BMAL1 controls PPARα and DBP, and REV-ERBα controls SHP and E4bp4 (Canaple et al., 2006; Duez et al., 2008). The circadian clock also regulates stability and activity of these factors. PER2 binding promotes ERα degradation, regulates PPARγ DNA binding, and co-activates PPARα-mediated transcription (Gery et al., 2007; Grimaldi et al., 2010; Schmutz et al., 2010), while CRY binding represses GR (Lamia et al., 2011). The clock can also regulate these factors indirectly, for instance, through their ligands such as glucocorticoid for GR (Reddy et al., 2007). The clock signal can also be mediated by transcription coactivators PGC-1α and BAF60a, or through post-transcriptional regulation by microRNAs (Gatfield et al., 2009; Tao et al., 2011; Wu et al., 2009).

1.2b Clock Regulation of the Epigenome

In addition to the linear genomic DNA sequences, information affecting the expression of individual genes can be encoded in the chromatin using mechanisms such as DNA methylation, histone modification and chromatin remodeling. This additional layer of gene regulation may be referred to as the epigenome. Epigenomic modification provides plasticity in gene expression and cellular functions in multi-cellular organisms, and allows reversible changes in response to changes in the their environment, including light, temperature, food availability and dietary composition which can affect many physiological processes, including development, aging, and metabolism (reviewed in Christensen and Marsit, 2011; Portela and Esteller, 2010).

Transcription factors control gene transcription by facilitating recruitment and activation of the transcription machinery, or by altering epigenome to recreate a more favorable environment for transcription, or most of the time both (reviewed in Farnham, 2009). Accumulating evidence, summarized in this section, implicates the core clock genes as partners of chromatin modifying enzymes (Table 1.1).

Histone acetylation Histone acetylation undergoes cyclic oscillation with the clock, as evident in mouse liver, not only at the promoters of the clock genes and but also in a genome-wide scale (Curtis et al., 2004; Etchegaray et al., 2003; Naruse et al., 2004; Ripperger and Schibler, 2006). Histone acetylation is controlled by a battle between HATs and HDACs, both of which have been shown to associate with the clock machinery and participate in clock-regulated rhythmic gene transcription.

The BMAL1/CLOCK or BMAL1/NPAS2 heterodimer recruits both HATs and HDACs. BMAL1 binds transcription coactivator p300 and possibly the CREB binding protein (CBP), while CLOCK and NPAS2 bind p300 and the CBP-associated factor (PCAF) (Curtis et al., 2004; Etchegaray et al., 2003; Takahata et al., 2000). All three coactivators have intrinsic HAT activity. In liver and heart, p300 exhibits a circadian association with CLOCK or NPAS2, correlating with increase in Per1 mRNA and histone H3 acetylation on the Per1 promoter (Curtis et al., 2004; Etchegaray et al., 2003). BMAL1/CLOCK also directly interacts with SIRT1, recruits SIRT1 to the Dbp gene in a timely manner, and drives rhythmic histone acetylation and gene transcription (Asher et al., 2008; Nakahata et al., 2008) (Figure 1.5). Moreover, CLOCK itself is a HAT, and its HAT activity is essential for the rhythmic acetylation at the Per1 promoter and the circadian expression of Per1 and Dbp (Doi et al., 2006).

PER and CRY direct the negative feedback signal to BMAL1/CLOCK probably through attenuating their affinity for DNA (Figure 1.1) (Ripperger and Schibler, 2006). In addition, through interaction with BMAL1/CLOCK, CRY1 can recruit Sin3B corepressor and HDAC1/2 to the Per1 promoter, and repress Per1 transcription through histone deacetylation (Naruse et al., 2004). Similarly, PER2 recruit Sin3A corepressor and HDAC1 through the polypyrimidine tract–binding protein–associated splicing factor (PSF) to the Per1 promoter (Duong et al., 2011).

REV-ERBs and RORs are members of the NR family, a family known to employ multiple coactivator and corepressor complexes for chromatin modification. REV-ERBα recruits nuclear receptor corepressor (NCoR) and HDAC3 to deacetylate histones and repress transcription (Yin and Lazar, 2005). RORs interact with both corepressors and coactivators (reviewed in Jetten, 2009), and the rhythmic binding of RORs to the Npas2 promoter are positively correlated with DNA accessibility and histone acetylation (Takeda et al., 2011).

Histone methylation Whereas histone acetylation is usually associated with transcriptional activation, methylation has a mixed effect on transcription, depending on the modification sites. CLOCK interacts with and recruits the mixed lineage leukemia 1 (MLL1), a HMT that specifically promotes H3K4me3, an activation mark. MLL1 is required for circadian H3K4 methylation and H3 acetylation and for circadian gene expression (Katada and Sassone-Corsi, 2010). PER1 associates with WD repeat-containing protein 5 (WDR5), and loss of WDR5 abolishes the circadian H3K4 and H3K9 methylation at the Rev-erbα promoter (Brown et al., 2005). Interestingly, MLL1 and WDR5 can be present in the same methyltransferase complex. BMAL1/CLOCK binds polycomb protein EZH2 and methylates H3K27 at the Per1 and Per2 promoters, which is essential for CRY-mediated transcriptional repression (Etchegaray et al., 2006). BMAL1/CLOCK also recruits JumonjiC and ARID domain-containing histone lysine demethylase 1a (JARID1a) to the Per2 promoter, though it appears that the circadian functions of JARID1a may be independent of its histone modifying activity (DiTacchio et al., 2011).

Chromatin Remodeling Chromatin remodeling often accompanies histone modification and transcriptional regulation (reviewed in Strahl and Allis, 2000). One study showed that RORs recruit the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex subunit BAF60a to drive rhythmic Bmal1 and G6pase expression (Tao et al., 2011). However, this aspect of clock function is understudied at the present time.

Rhythmic chromatin modification is not limited to the promoter of clock genes that have been studied, as ChIP-seq revealed that BMAL1 binds thousands of sites throughout the genome (Dufour et al., 2011; Rey et al., 2011). In addition, the clock can drive rhythmic epigenomic programming indirectly through clock-regulated transcription factors, such as NRs, which then recruit HATs and HDACs.

Non-histone targets of clock-associated histone modifiers Histone modifying enzymes also target transcription factors, cofactors and enzymes. CLOCK acetylates its partner BMAL1, and facilitates CRY1 binding and CRY1-mediated transcriptional repression (Hirayama et al., 2007). CLOCK also acetylates GR, attenuates its DNA binding, and regulates glucocorticoid response (Charmandari et al., 2011; Nader et al., 2009). SIRT1 counteracts CLOCK-mediated BMAL1 acetylation, deacetylates and degrades PER2 thus derepressing BMAL1/CLOCK (Asher et al., 2008; Nakahata et al., 2008) (Figure 1.5). Activation or inhibition of transcription activity will also alter the epigenomic state of the

target genes. Also clock-associated histone modifiers can be recruited by other transcription factors and function independent of the clock machinery.

1.3 REV-ERBα Is an Orphan Nuclear Receptor

1.3a Nuclear Receptor

REV-ERBα is a member of the nuclear receptor superfamily. Nuclear receptors are a large family of transcription factors that regulate differentiation, development and metabolic homeostasis in animals (reviewed in Bain et al., 2007). Classical nuclear receptors are ligand activated, and mediate the effects of important hormones, such as estrogen, glucocorticoid and thyroid hormone.

Structure of nuclear receptors All members of the nuclear receptor superfamily share a similar structure. From N-terminal to C-terminal, a nuclear receptor can be divided into four domains: the N-terminal A/B domain, the DNA binding domain (DBD), the hinge region and the ligand binding domain (LBD) (reviewed in Bain et al., 2007). Both DBD and LBD are conserved among the family. Crystal structures show that LBD is a globular domain made up of approximately 12 α-helices (reviewed in Li et al., 2003). LBD contains a hydrophobic ligand binding pocket and a ligand-regulated transcriptional activation function 2 (AF-2). AF-2 consists of Helix 12 and several other helices, and provides a hydrophobic groove that interacts with transcription coactivators (Bledsoe et al., 2002; Shiau et al., 1998). In addition, LBD is required for dimerization of nuclear receptors. DBD, as indicted by its name, recognizes and binds specific DNA sequences, known as the response elements. The core response element is usually a hexanucleotide

sequence, known as the half site. DBD is connected to LBD through the hinge region. The N-terminal A/B domain contains another transcriptional activation function termed as AF-1, and has weak conservation in the family. The variable N-terminal confers specificity to the functions of different nuclear receptors (Figure 1.3).

Classification of nuclear receptors The nuclear receptor superfamily is usually divided into three different classes, based on their ligands and DNA binding properties (reviewed in Bain et al., 2007). The first class is steroid receptors, including androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR). In the presence of the ligand, steroid receptors dissociate from heatshock proteins, translocate to the nucleus from cytosol, and bind the palindromic half sites as a homodimer. The second class is the thyroid/retinoid receptor family, including thyroid receptor (TR) and retinoic acid receptor (RAR). Unlike the first class, the thyroid/retinoid receptor family stays on the genome, and ligand binding induces conformational changes in Helix 12, allowing the interaction with coactivators. They also have different dimerization patterns, and many of them form heterodimer with retinoid X receptor (RXR). Moreover, their response elements contain direct repeats of the half sites instead of palindromic sequences. The last class is known as the orphan nuclear receptor, which contains REV-ERBα, its mammalian paralog REV-ERBβ and the RAR-related orphan receptors (RORs). Most nuclear receptors in this class have no known cognate ligands, and they were identified based on sequence homology to known nuclear receptors. Recent studies identified the ligands of some of these orphan nuclear receptors, such as bile acids for farnesoid X receptor (FXR) and fatty acids for peroxisome proliferator-activated receptors (PPARs).

These receptors are thus considered adopted orphan receptors (reviewed in Sonoda et al., 2008).

Coregulators of nuclear receptors Nuclear receptors regulate transcription through recruiting coactivator and corepressor complexes. Coactivator complexes usually contain histone acetyltransferases (HATs), such as p300 and CBP, and chromatin remodeling enzymes, such as BRG1, which create an epigenomic environment that favors gene transcription. Coactivators also help recruit RNA polymerase complex, and directly promote transcription (reviewed in Hermanson et al., 2002). Corepressor complexes usually contain histone deacetylases (HDACs), such as HDAC3 in the nuclear receptor corepressor (NCoR) complex and HDAC1/2 in the nucleosome remodeling and deacetylation (NURD) complex (reviewed in Perissi et al., 2010). In addition, corepressor complexes also contain chromatin remodelers, histone methyltransferases and demethylases.

1.3b REV-ERBα

REV-ERBα was one of the first orphan nuclear receptor to be discovered (Lazar et al., 1989; Miyajima et al., 1988). Later, a highly related nuclear receptor REV-ERBβ was discovered, as well as a non-mammalian homologue E75, which is present in *Drosophila* and *C. elegans* (Bonnelye et al., 1994; Dumas et al., 1994; Retnakaran et al., 1994; Segraves and Hogness, 1990). Rev-erbα is highly expressed in fat, muscle and brain, and regulates development in these tissues (Chawla and Lazar, 1993; Chomez et al., 2000; Downes et al., 1995).

REV-ERBα isoforms REV-ERBα has multiple isoforms. In human and rat, there are two different isoforms of Rev-erbα mRNA, transcribed from different promoters. The protein products of these two isoforms differ in their N-terminal domain (Triqueneaux et al., 2004). The longer isoform, REV-ERBα1, is 614 amino acids long. The shorter isoform REV-ERBα2, with N-terminal encoded in a different first exon, is 106 amino acid shorter. The importance of having two different isoforms remains unclear (Adelmant et al., 1996; Chopin-Delannoy et al., 2003).

REV-ERBα is a transcription repressor REV-ERBα recognizes the classical nuclear receptor half site, a hexanucleotide AGGTCA sequence. Unlike many nuclear receptors, REV-ERBα does not dimerize with RXR. It can bind the half site preceded by an AT-rich sequence at the 5' end as a monomer, or a direct repeat of the half sites separated by two nucleotides, known as the DR2 element as a homodimer (Harding and Lazar, 1993). Alternatively, two REV-ERBα can bind independently at two half sites spaced apart, and collaboratively mediate transcriptional repression, as demonstrated in the promoter of the Bmal1 gene (Yin and Lazar, 2005).

REV-ERBα lacks Helix 12, which is responsible for coactivator recruitment. In consistent with its structure, it primarily functions as a transcription repressor by recruiting the NCoR corepressor complex. REV-ERBα interacts with NCoR through the CoRNR motif located on the N-terminal of NCoR, and it prefers NCoR over other corepressors, including the closely related SMRT (silencing mediator of retinoic acid and

thyroid hormone receptor) (Hu et al., 2001; Ishizuka and Lazar, 2003). Importantly, REV-ERBα interacts with NCoR with a stoichiometry of two REV-ERBαs to one NCoR, as determined by steric preference of their binding interface (Zamir et al., 1997). Therefore, REV-ERBα can only recruit NCoR and conduct active repression when two REV-ERBαs bind two half sites as a homodimer. When REV-ERBα binds as a monomer, it may repress transcription passively by competing with transcription activators, such as nuclear receptor RORs (Figure 1.4).

The transcription repression activity of NCoR derives from its ability to directly interact with and inhibit the basic transcriptional machinery and its ability to recruit and activate histone deacetylase (HDAC) (reviewed in Hu and Lazar, 2000). Although NCoR has been shown to interact with many HDACs, its deacetylase activity mainly depends on HDAC3, which we will discuss later (Guenther et al., 2000; Ishizuka and Lazar, 2003; Li et al., 2000; reviewed in Lazar, 2003). Thus HDAC3 is required for transcription repression by nuclear receptors, such as TR and REV-ERBα (Ishizuka and Lazar, 2003). Remarkably, the enzyme activity of HDAC3 requires NCoR, which interacts with HDAC3 through its deacetylase activation domain (DAD) (Guenther et al., 2001).

REV-ERBα is a member of the core clock machinery REV-ERBα was identified as a major regulator of the cyclic expression of Bmal1, which is an essential component of the basic clock machinery. Two REV-ERBα half sites were found in the promoter of Bmal1, and REV-ERBα binding to the Bmal1 promoter is inversely correlated with Bmal1 expression in a 24h cycle. Genetic ablation of Rev-erbα severely blunted the circadian

expression of Bmal1, and significantly shortens the circadian period. REV-ERBα was also shown to repress other clock genes, Clock, Cry1 and Per2, and was long known to be self-regulated (Adelmant et al., 1996; Preitner et al., 2002; Ueda et al., 2002). As a major repressor of Bmal1, REV-ERBα is transcriptionally activated by BMAL1/CLOCK, forming a negative feedback loop that drives the circadian expression of both Rev-erbα and Bmal1 (Triqueneaux et al., 2004).

The role of REV-ERBα in the circadian clock has long been considered auxiliary due to the generally intact circadian rhythm in Rev-erbα deficient mice (Preitner et al., 2002). However, the basic clock machinery is featured by the redundancy of its components: Bmal1/Bmal2, Clock/Npas2, Per1/Per2/Per3 and Cry1/2. Except for Bmal1, the loss of any single component can be compensated by the remaining ones, and the circadian rhythm is largely preserved (Dallmann et al., 2011; van der Horst et al., 1999; Shi et al., 2010; Zheng et al., 2001). Similar to other clock components, REV-ERBα has a twin brother, REV-ERBβ, whose role in circadian rhythms remains unclear. It has been suspected that REV-ERBβ compensates for the loss of REV-ERBα, and the role of both REV-ERBs in circadian clocks deserve a careful look.

REV-ERBα links the circadian clock and metabolism In addition to its role in circadian clock, REV-ERBα also emerged as an important regulator of several metabolic pathways, including lipid and cholesterol metabolism. REV-ERBα represses apolipoprotein ApoA-I and ApoC-III, and genetic ablation of Rev-erbα leads increased plasma triglycerides and TG-rich very low density lipoprotein (VLDL) particles in mouse (Raspé et al., 2002; Vu-

Dac et al., 1998). REV-ERBα may also regulate fatty acid synthesis through the very long chain fatty acid elongase Elovl3 and peroxisome β-oxidation through enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) (Anzulovich et al., 2006; Kassam et al., 1999). As discussed in **Session 1.1c**, REV-ERBα also regulates bile acid synthesis and adipocyte differentiation. As a result of these findings, REV-ERBα is believed to be part of the missing link between the circadian clock and the circadian metabolic activities.

Post-translational regulation of REV-ERBα REV-ERBα can be phosphorylated by GSK3β at its N-terminal, and phosphorylation protects REV-ERBα from ubiquitin-mediated degradation by ARF-BP1 and PAM. Lithium is a potent inhibitor of GSK3β activity, and thus promotes REV-ERBα degradation and activates Bmal1transcription (Yin et al., 2006, 2010). Originally categorized as an orphan receptor, REV-ERBα was recently shown to bind heme. Heme can enhance NCoR recruitment and transcription repression by REV-ERBα (Yin et al., 2007). The significance of this regulation will be discussed later.

1.4 Metabolic Feedback to the Clock

The circadian clock is regulated by metabolic signals such as fasting/feeding and dietary factors. Fasting and refeeding regulates the clock gene expression in peripheral tissues, particularly liver (Kawamoto et al., 2006; Tahara et al., 2011). High fat diet (HFD) lengthens the circadian period and attenuates clock oscillation (Kohsaka et al., 2007). Clock-associated histone modifiers such as p300 and SIRT1 are also regulated. Fasting decreases p300 phosphorylation and activates p300, and induces Sirt1 expression, while

high fat diet (HFD) inhibits p300 phosphorylation, lengthens the circadian period and attenuates clock oscillation (Liu et al., 2008b; Rodgers et al., 2005). The impact of metabolism might be mediated through hormones, such as glucagon and insulin (Liu et al., 2008b; Tahara et al., 2011), as well as metabolites. The circadian clock can sense the intracellular metabolite levels, adjust its own rhythm and functions and allows a fine and precise temporal regulation of metabolic pathways within individual cells. We will focus on the key metabolites such as NAD^+, AMP and heme, though other metabolites may also play a role.

NAD^+/NADH NAD^+/NADH redox equilibrium indicates the metabolic state of the cell. NADH and NADPH directly binds CLOCK or NPAS2 and enhances BMAL1/CLOCK (or NPAS2) DNA binding, while the reduced form NAD^+ binds and inhibits (Rutter et al., 2001). NAD^+/NADH ratio also regulate SIRT1, which deacetylates PER2, BMAL1 and histones (Asher et al., 2008; Nakahata et al., 2008) (Figure 1.5). Intracelluar NAD^+ levels are circadian, and are critical for circadian clock functions (Sahar et al., 2011).

NAD^+ levels are determined both by the fasting/feeding cycles and circadian rhythms. Fasting induces NAD^+ levels in liver, as well as SIRT1 protein levels (Rodgers et al., 2005). Circadian clocks regulates NAD^+ mainly through the NAD^+ salvage pathway, in which NAD^+ is synthesized from nicotinamide (NAM), the byproduct of sirtuins, through the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT). BMAL1/CLOCK drives the circadian expression of NAMPT and contributes to the rhythmic NAD^+ levels. NAD^+ activates while NAM represses SIRT1, which interacts

with BMAL1/CLOCK and inhibits NAMPT transcription. Therefore, NAD^+ drives a negative feedback on its own synthesis through SIRT1 and the circadian clock, and through NAD^+, the intracellular metabolic state can regulate the clock (Figure 1.5) (Nakahata et al., 2009; Ramsey et al., 2009).

NAD^+ is also a substrate of poly(ADP-ribose) polymerase 1 (PARP-1), an NAD^+-dependent ADP-ribosyltransferase. PARP-1 activity oscillates in a daily manner, and is regulated by feeding. PARP-1 binds and poly(ADP-ribosyl)ates clock at the onset of the light phase, regulates BMAL1/CLOCK interaction with PER and CRY and BMAL1/CLOCK DNA binding, and mediates food entrainment of the liver clock. NAD^+ might play a role in regulating PARP-1 activity by feeding, but other mechanisms are also involved (Asher et al., 2010).

AMP/ADP AMP and ADP bind AMPK, inhibit AMPK dephosphorylation and activate its kinase activity. Therefore, AMPK functions as a sensor of energy state in the cell, which is reflected by AMP and ADP levels (Cantó and Auwerx, 2011). Several studies place AMPK as a circadian clock regulator (Lamia et al., 2009; Um et al., 2007, 2011; Vieira et al., 2008) with two different mechanisms: AMPK activates CKIε and promotes PER2 degradation(Um et al., 2007); AMPK phosphorylates Cry1 and destabilizes it (Lamia et al., 2009) (Figure 1.5). AMPK also regulates the NAD^+/NADH ratio, through which it crosstalks with SIRT1 (Cantó et al., 2009).

Heme As we discussed earlier, heme is a ligand of REV-ERBα and REV-ERBβ, and enhances corepressor recruitment and transcriptional repression (Pardee et al., 2009; Phelan et al., 2010; Raghuram et al., 2007; Yin et al., 2007) (Figure 1.5). Through heme, REV-ERBs can sense the redox state and gas such as O_2, NO and CO, as they regulate REV-ERB structure and corepressor recruitment (Pardee et al., 2009). Also heme binding can be regulated by the redox state of the REV-ERBβ protein (Gupta and Ragsdale, 2011). Heme is also a component of NPAS2, whose heterodimeric DNA binding with Bmal1 is inhibited by CO (Dioum et al., 2002; Gilles-Gonzalez and Gonzalez, 2004).

Like NAD^+, heme can also negatively regulate its own synthesis through the circadian clock. The control is exerted on the expression of the rate-limiting enzyme in heme biosynthesis, δ-aminolevulinate synthase (ALAS1), whose expression is circadian. Heme inhibits BMAL1/NPAS2-activated ALAS1 expression and heme biosynthesis (Kaasik and Lee, 2004). In parallel, through REV-ERBα, heme represses PGC-1α transcription, a potent inducer of heme biosynthesis (Wu et al., 2009) (Figure 1.5). Both NAD^+ and heme are critical metabolites and indicators/sensors of the metabolic state in the cells. The negative metabolite feedback loops connect metabolism with the circadian clock, and are critical for metabolic homeostasis and fine tuning of the clock function.

1.5 Rhythmic Regulation of Metabolism by Food Intake

A key unanswered question is whether the rhythmic feature of metabolism is a passive response to the fasting/feeding cycles in metabolic tissues or an active anticipatory mechanism directly governed by the circadian clock. Recent transcriptomic studies in

mouse liver provide some insight. Liver-specific overexpression of REV-ERBα abolishes oscillation of almost all the rhythmic transcripts found in wildtype mice, suggesting that they are controlled by the liver clock (Kornmann et al., 2007). Meanwhile, only a small subset of these transcripts, including the clock genes, maintain their oscillation under fasting, suggesting most of the rhythmic transcripts are also regulated by fasting/feeding. Interestingly, in clock deficient mice restricted feeding can resume oscillation of about 60% of the rhythmic transcripts and even increase their amplitude, along with many other non-rhythmic genes (Vollmers et al., 2009). These observations suggest that under normal conditions, peripheral clocks and fasting/feeding cycles work together to drive rhythmic gene expression and metabolic activities.

Fasting/feeding activities are usually aligned with sleep/wake cycles, and are timed by the central clock. Therefore, normally both the central clock and peripheral clocks synergize in rhythmic metabolic regulation. In addition to feeding/fasting, the central clock can also drive metabolic activities through hormones and body temperature (Brown et al., 2002; Reddy et al., 2007). For instance, blood glucocorticoid levels exhibits circadian oscillation (Oster et al., 2006). In the absence of the central clock, daily glucocorticoid injection can restore about 60% of the liver circadian transcriptome, probably through hepatocyte nuclear factor 4α (HNF4α) (Maywood et al., 2007; Reddy et al., 2007). Therefore, under normal conditions, the central clock synchronize peripheral clocks, fasting/feeding cycles, hormone secretion and body temperature changes, and all of these contribute to the rhythmic metabolic activities throughout the body (Figure 1.2).

On the other hand, fasting/feeding is also subject to food availability in the environment, and changes in metabolic profiles as induced by fasting/feeding can impact and even reset the peripheral clocks in metabolic tissues. Thus, in addition to the clock regulating metabolism, metabolism can regulate the clock.

1.6 Hypothesis and Objectives

In mammals, metabolic processes in peripheral organs display robust circadian rhythms, coordinated with the daily cycles of light and nutrient availability. Circadian misalignment causes metabolic dysfunction, and people engaged in night-shift work suffer from higher incidences of obesity, diabetes, and metabolic syndrome. Transcriptomics evidence suggests that circadian control of metabolism is achieved through transcriptional and probably epigenomic regulation of metabolic genes. In this dissertation, we explore the role of histone deacetylase HDAC3, an epigenomic modifier, in the circadian regulation of hepatic metabolism. First we hypothesize that HDAC3 displays circadian genomic binding in mouse liver, and drives rhythmic histone acetylation and thus oscillation of important metabolic genes. HDAC3 is thus critical for metabolic homeostasis in liver. To address this hypothesis, in Chapter 3, we profile HDAC3 binding and histone acetylation in mouse liver genome at different times of the day, and reveal the role of REV-ERBα in regulating HDAC3 genomic binding. Next, we hypothesize that other nuclear receptors, particularly the highly related REV-ERBβ and RORs, also play an important role in the circadian regulation of hepatic metabolism. In Chapter 4, we examine how these nuclear receptors work with REV-ERBα and HDAC3

in regulating circadian rhythms and metabolism. Chapter 5 summarizes the work and discussed the future directions.
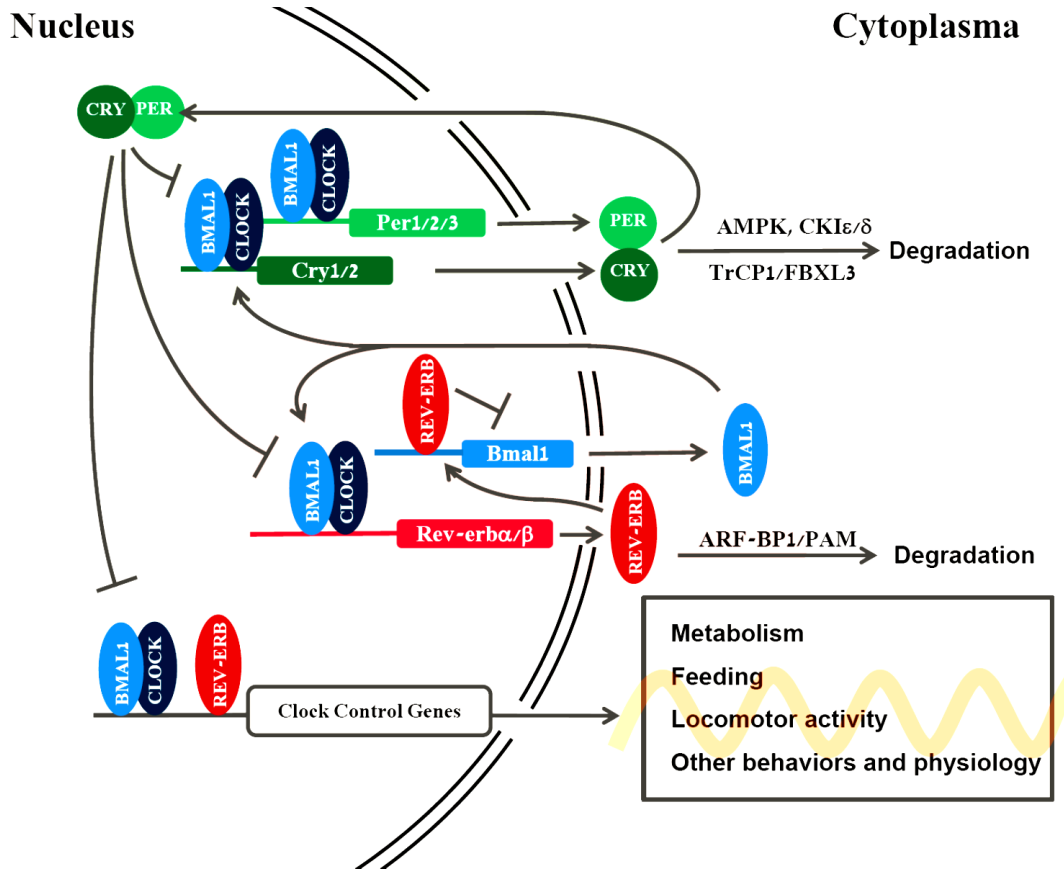
Figure 1.1 The Core Machinery in Mammals. The core clock machinery consists of two transcriptional-translational feedback loops. In the first loop, BMAL1/ClOCK activates transcription of Per and Cry, whose proteins bind and repress BMAL1/CLOCK activity. A second loop, once considered auxiliary, controls Bmal1 expression; BMAL1/CLOCK activates Rev-erbα and Rev-erbβ transcription, and REV-ERB proteins repress Bmal1 transcription. The core clock proteins are also subject to post-translational regulation by AMPK, CKIε/δ, TrCP1/FBXL3 and ARF-BP1/PAM. BMAL1/CLOCK and REV-ERBs also control the expression of output genes, driving circadian rhythms in metabolism, feeding, locomotor activity and others.
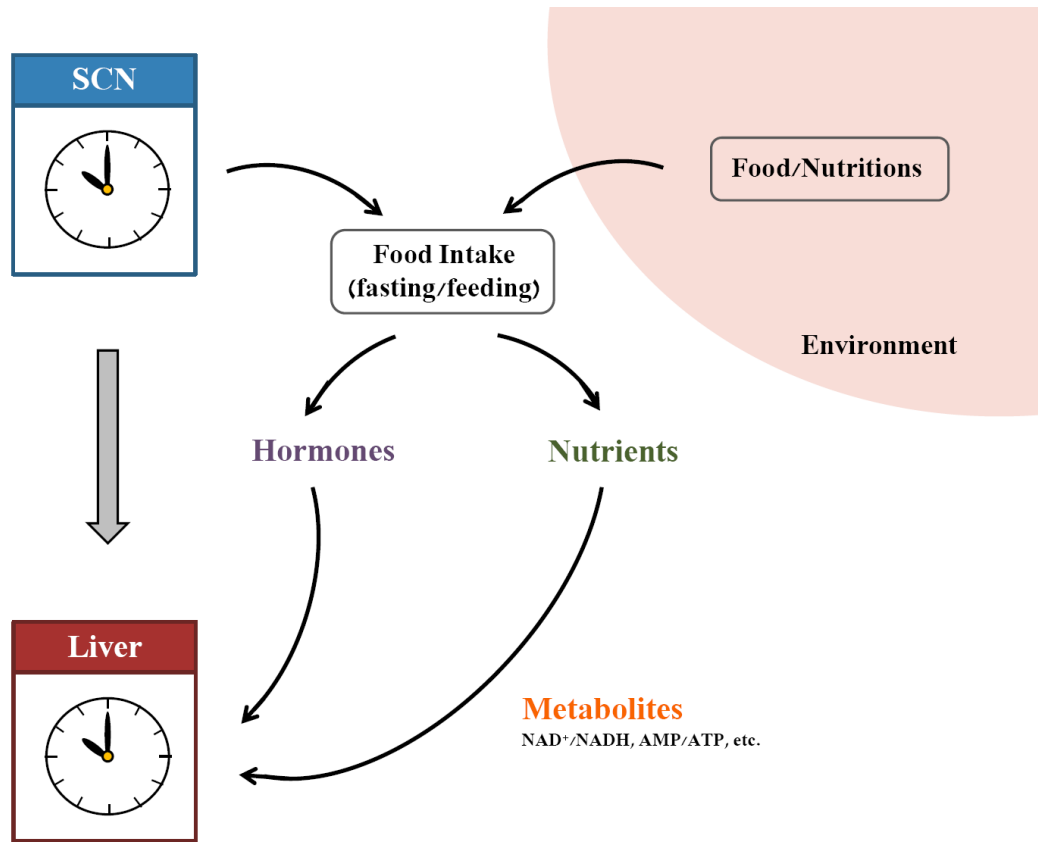
Figure 1.2 Assembly of the Circadian Clocks in Mammals. The circadian clocks in mammals are assembled in a hierarchical manner. The central clock, located in the SCN in hypothalamus, regulates and synchronizes the peripheral clocks located in many metabolic organs including liver through neuronal and hormonal signals (Grey arrow). However, the predominant Zeitgeber for the liver clock is food intake, which regulates the liver clock and metabolic rhythms through hormonal and nutritional signals. When restricted food availability shifts the fasting/feeding cycles, peripheral clocks can be reset separately from the central clock.

Figure 1.3 Schematic Structure of Nuclear Receptor. A typical nuclear receptor can be divided into four domains: the N-terminal A/B domain, the DNA binding domain (DBD), the hinge region (H) and the ligand binding domain (LBD). The LBD binding domain contains activation function 2 (AF-2), which mediates coactivator recruitment and transcriptional activation. The A/B domain contains activation function 1 (AF-1).
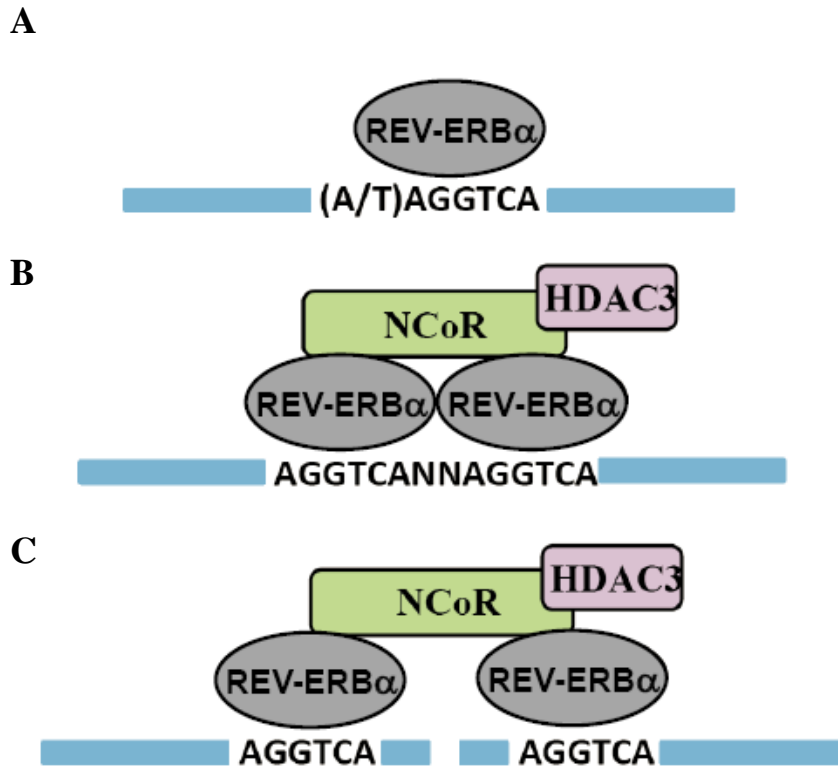
Figure 1.4 REV-ERBα-Mediated Transcription Repression.

A. REV-ERBα binds as a monomer at a nuclear receptor half site (AGGTCA) preceded by an A/T-rich sequence, and does not recruit corepressors.

B. REV-ERBα binds as a homodimer at the DR2 element (direct repeat of half sites separated by 2 nucleotides) and recruits the NCoR corepressor complex and HDAC3.

C. REV-ERBα binds independently at two half sites spaced apart, but cooperatively recruits the NCoR corepressor complex and HDAC3.
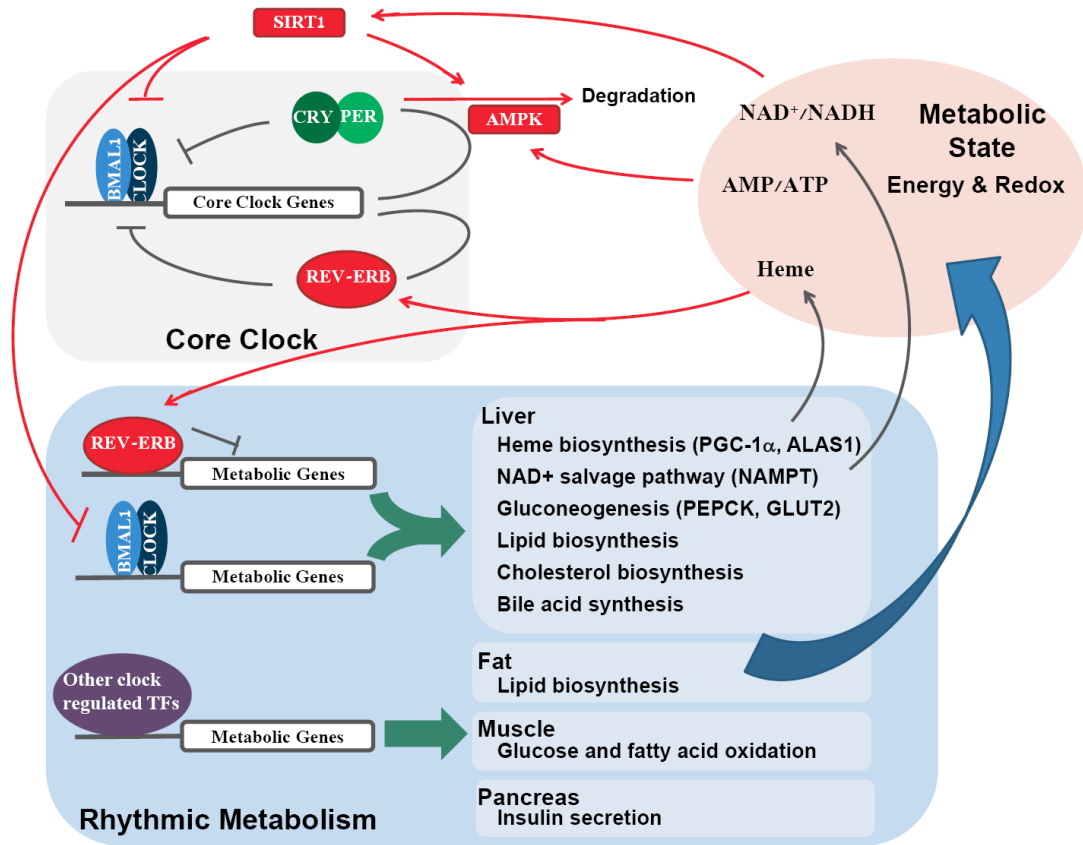
Figure 1.5 Metabolite Regulation of the Clock. BMAL1/CLOCK and REV-ERBs drive rhythmic metabolic outputs, including NAD^+ and heme biosynthesis, while intracellular NAD^+ and heme feedback on the clock through their sensors, SIRT1 and REV-ERBs, respectively. Intracelluar AMP levels regulates the circadian clock through activation of AMPK and degradation of PER and CRY. The core clock also drives many metabolic pathways in different tissues, which also contribute to the intracellular metabolite pool and metabolic state.

Table 1.1 Clock-associated Chromatin Modifying Activities

Clock Component	Associated Chromatin Modifier	Targets	References
BMAL1 /CLOCK	Clock	H3K9, H3K14 Bmal1(K537), GR	(Doi et al., 2006; Nader et al., 2009; Charmandari et al., 2011)
	p300	H3	(Etchegaray et al., 2002; Takahata et al., 2000)
	PCAF		(Curtis et al., 2004)
	CBP		(Takahata et al., 2000; Curtis et al., 2004)
	SIRT1	H3K9, H3K14, Bmal1(K537), Per2	(Asher et al., 2008; Nakahata et al., 2008)
	MLL1	H3K4	(Katada and Sassone-Corsi, 2010)
	EZH2	H3K27	(Etchegaray et al., 2006)
	JARID1a		(DiTacchio et al., 2011)
BMAL1 /NPAS2	p300	H3, H4	(Curtis et al., 2004)
	PCAF	H3, H4	(Curtis et al., 2004)
	ACTR		(Curtis et al., 2004)
PER	PSF-Sin3A-HDAC1	H3, H3K9, H4K5	(Duong et al., 2011)
	WDR5	H3K4	(Brown et al., 2005)
CRY	Sin3B-HDAC1/2	Histone H3, H4	(Naruse et al., 2004)
REV-ERBα,β	NCoR-HDAC3	H3, H4, H3K9	(Ishizuka and Lazar, 2003; Yin and Lazar, 2005; Feng et al, 2011; Bugge et al, 2012)

Chapter 2

Material and Methods

Animals

WT C57Bl/6 mice were purchased from Jackson Labs. The Rev-erbα knockout mice were obtained from B. Vennström, and backcrossed ≥7 generations with C57/Bl6 mice. The HDAC3^{fl/fl} mice were produced from C57Bl/6 embryonic stem cells with loxP sites flanking exon 4 to exon 7, which encode the catalytic domain of HDAC3 (Mullican et al., 2011). The HDAC3^{fl/fl} mice were then crossed with Alb-Cre mice to generate HDAC3^{fl/fl}/Alb-Cre mice. The RORα sg/sg mice was purchased from Jackson Labs.

8 to 12 week old WT and mutant male mice were housed under the 12h-light/12h-dark (LD) cycles (lights on at 7 AM, lights off at 7 PM) for at least 2 weeks, and euthanized at ZT10 (5 PM) or ZT22 (5 AM). For constant darkness, light remained off after 12h darkness, and mice were euthanized either 10h later (CT10) or 22h later (CT22). For restricted feeding, mice were kept in LD cycles for 2 weeks with food available only between ZT3 and ZT11, and euthanized in constant darkness at CT10 or CT22. All the animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Liver tissues from the chronic jet-lag mice and the Cry DKO mice were a gift from Dr. Loning Fu at the Baylor College of Medicine.

Synchronized MEFs

MEFs were collected at embryonic day 13.5 from wild-type or Rev-erbα-null embryos, expanded, and seeded in 24-well plates at a density of 5×10^4 cells per well. The MEFs

were washed in PBS and incubated for 20 h with 5.7×10^9 particles (GC) of adenovirus per well in DMEM containing 0.5% bovine serum. Serum shock was performed by replacing starvation medium with 50% horse serum for 2 h. Following two washes in PBS, cells were changed back to DMEM containing 0.5% bovine serum, and total RNA was harvested at the indicated time points.

Antibodies and reagents

HDAC3 antibodies were purchased from Abcam (ab7030, for ChIP-Seq and western blot) and Santa Cruz (sc-11417, for validation). REV-ERBα antibody was purchased from Cell Signaling Technology (#2124). REV-ERBβ antibody was raised in a rabbit against amino acids 243–261 (Covance). Acetylated H3K9 antibody was purchased from Millipore (#07-352). Two RORα antibodies were purchased from Santa Cruz: C-16 (sc-6062) for ChIP-seq and H-65 (sc-28612) for validation. p300 antibody was purchased from Santa Cruz (sc-585).

Constructs and gene transductions

The adeno-associated viruses encoding Cre recombinase (AAV-Cre) and GFP (AAV-GFP) were obtained from the Vector Core of the Penn Diabetes and Endocrinology Research Center. The Cre or GFP gene was inserted into the AAV2/8 vector containing the liver specific Tbg promoter (Gao et al., 2002). Each 12 week old male HDAC3$^{\text{fl/fl}}$ mouse received 1.5×10^{11} particles of virus through tail-vein injection. The adeno-associated virus encoding mouse RORα4 was constructed using the same AAV2/8 vector containing Tbg promoter as AAV-GFP. The virus was packaged by the Vector Core of

the Penn Diabetes Research Center. Each mice received 4×10^{11} particles (GC) of virus through tail vein injection. The adenoviruses encoding shRNA targeting the β-galactosidase (TGCACCTGGTAAATCTTAT) or the Rev-erbβ gene (GCACTAAGGACCTTAATAATG) were constructed using the BLOCK-iT adenoviral RNAi expression system from Invitrogen (#K4941-00) and subsequently amplified and purified by the Vector Core of the Penn Diabetes Research Center. Each mouse received 5.7×10^{11} particles (GC) of virus through tail vein injection.

Chromatin Immunoprecipitation (ChIP)

Mouse liver was harvested immediately after euthanasia. It was quickly minced and cross-linked in 1% Formaldehyde for 20 min, followed by quenching with 1/20 volume of 2.5 M glycine solution, and two washes with $1\times$ PBS. Nuclear extracts were prepared by Dounce homogenization in cell lysis buffer (5mM PIPES, 85mM KCl, 0.5% Igepal and complete protease inhibitor tablet from Roche, pH8.0). Chromatin fragmentation was performed by sonication in ChIP SDS lysis buffer (50 mM HEPES, 1% SDS, 10 mM EDTA, pH7.5), using the Bioruptor (Diagenode) for the HDAC3, Rev-erbα and RNA Pol II ChIP, or by MNase (Sigma) digestion (6U/ml for 20 minutes) in MNase digestion buffer (20mM HEPES, 100mM NaCl, 1.5mM $CaCl_2$, 3mM $MgCl_2$ and 1mM PMSF, pH7.5) for H3K9 acetylation. Alternative: Frozen mouse liver was ground in liquid nitrogen, and cross-linked; After wash, the cross-linked liver was directly sonicated in ChIP dilution buffer (50 mM HEPES, 155 mM NaCl, 1.1% Triton X-100, 0.11% Na-deoxycholate, 0.1% SDS, and complete protease inhibitor tablet, pH7.5). Proteins were immunoprecipitated in ChIP dilution buffer using different antibodies. Cross-linking was

reversed overnight at 65°C, and DNA isolated using phenol/chloroform/isoamyl alcohol.

Precipitated DNA was analyzed by quantitative PCR.

Quantitative PCR

Quantitative PCR was performed with Power SYBR Green PCR Mastermix and the

PRISM 7500 instrument (Applied Biosystems), and analysis was performed by the

standard curve method. Primer sequences used for ChIP-PCR analysis can be found in

Supplemental Table 2.

ChIP-seq

For each factor, ChIP was performed independently on liver samples from 4 or 5

different mice, and the precipitated DNA or input DNA samples were pooled. 10ng of the

pooled DNA were then amplified according to ChIP Sequencing Sample Preparation

Guide provided by Illumina, using enzymes from New England Biolabs and PCR

Purification Kit and MinElute Kit from Qiagen. Deep sequencing was performed by the

Functional Genomics Core (J.Schug and K. Kaestner) of the Penn Diabetes

Endocrinology Research Center using the Illumina Genome Analyzer IIx, and sequences

were obtained using the Solexa Analysis Pipeline.

ChIP-seq peak calling and data normalization

The sequences identified were mapped to the mouse genome (NCBI36/UCSC mm8)

using BOWTIE software (Langmead et al., 2009). Only the sequences uniquely mapped

with no more than 2 mismatches in the first 28 bp were kept and used as valid reads. Peak

calling for HDAC3, NCoR and REV-ERBα was carried out by MACS (version 1.4.0b, allowing multiple reads aligning at the same location; options --mfold 10,30 --pvalue 1e-5), on each ChIP-seq file against the matching input file (Zhang et al., 2008a). H3K9Ac ChIP-seq data was processed by NPS (Zhang et al., 2008b), applying the wavelet denoising to define H3K9ac enriched well-positioned nucleosomes. The signal files generated from MACS or NPS (aligned reads encompassing each locus), were normalized to the sequencing depth (per million total reads). Except for the H3K9Ac data, PeakSplitter (Salmon-Divon et al., 2010) was further applied to split the nearby peaks and find the peak summits by using the normalized signal files, limiting the height of peak summit to a minimum of 1. The peak summits were used as the binding site centers, and the normalized signal files were used as the binding strength for further analysis.

Binding sites annotation and profiling

CEAS (Cis-regulatory Element Annotation System) (Shin et al., 2009) was applied to calculate the enrichment of the binding sites in the promoter, exon, intron, UTR and other genomic regions against the mappable mouse genome using the binomial model. CEAS was also used to generate the aggregation plot of the normalized binding signals at ± 1 kb surrounding site centers at 10 bp resolution. The binding signals of different factors and conditions surrounding each site centers are clustered by k-means clustering with the k parameter for the number of clusters set to 7. The kmeans function in R is used and the centers for each cluster are sorted before the heatmap is drawn. The heatmap color scale indicates the binding signals per million total reads for individual factors. The heatmap of

Pol II ChIP-seq signals was aligned at transcription start sites considering the orientation of genes.

Motif analysis

To find the enriched DNA motifs at HDAC3 binding site centers, a *de novo* motif scan by MDscan algorithm (Liu et al., 2002) and a search against JASPAR motif collection were performed at 600 bps windows around site centers. The motifs were further adjusted according to the distance from motif locations to the HDAC3 binding site centers. Details of the algorithm are as follows:

Known DNA motifs that are enriched relative to the center of ChIP-seq sites were identified using the following statistic. All sites were trimmed or expanded to 600 bp centered at the middle point of the identified ChIP-enriched regions. All subsequences within the designated window from the site centers were scored by a JASPAR motif or a *de novo* motif from MDscan algorithm to identify hits with a relative entropy cutoff t. Let x_i, a value between 0 and 1, denote the relative location of motif hit i on the ChIP regions, where 0 is the center of the ChIP region and 1 the edge. Out of N total motif hits, we define a z-score, $\sum_{i=1}^{N}(x_i - 0.5)/\sqrt{N/12}$ to assess the positional bias of a motif towards the center of the regions. Different integer cutoffs $t \geq 3$ were tested for each motif, and the cutoff resulting from the highest z was selected. This statistic is based on the assumptions that non-significant DNA motifs will be uniformly distributed across the ChIP regions and the null distribution of $\sum_{i=1}^{N} x_i$ can be estimated as the N-fold convolution of uniform density functions.

Gene expression microarray

Liver samples were taken from the HDAC3$^{fl/fl}$ mice 1 week after injection of AAV-Cre or AAV-GFP virus (4 mice per group). Total RNA was extracted from each liver sample using the RNeasy Mini Kit (Qiagen). Each RNA sample was processed with the Ambion WT Expression Kit and the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix), and hybridized to the Mouse Gene 1.0 ST Array (Affymetrix). The array was then read by GCS3000 laser scanner (Affymetrix), and microarray data analysis was carried out using Partek® Genomics Suite software (Copyright, Partek Inc.). Data from 8 samples were subjected to background subtraction, quantile normalization, log2 transformation and probeset summarization using RMA (Robust Multichip Average) algorithm. Only the probe sets that interrogate genes were retained and those meant for QC and normalization purposes were excluded from further analysis. Within Partek, a 1-way ANOVA using genotype as the factor was run on the data to obtain p-values for differential expression of genes between the knock-out and WT mice. The p-values were adjusted for step-up p-value or False Discovery Rate (FDR) for each gene. Fold-change was multiple testing using the Benjamini-Hochberg step-up method. The gene expression microarray and subsequent data analysis were performed by Penn Microarray Core. All mouse RefSeq genes were ranked based on their expression index in the HDAC3$^{fl/fl}$ mice injected with AAV-GFP (considered as WT). The top 10%, middle 10% and bottom 10% were selected for HDAC3 association study. Genes up-regulated or down-regulated in the AAV-Cre injected mouse were selected with a maximum p-value 10^{-3} or a minimum absolute fold-change of 1.2. The circadian gene list was obtained from CIRCA website

40

(Hughes et al., 2009), with a maximum COSPOT q-value of 0.001. Based on this list, all known genes were divided into circadian genes and non-circadian genes for HDAC3 association study. The Rev-Erbα knockout expression data were downloaded from ArrayExpress (E-TABM-726) (Le Martelot et al., 2009).

Association study of HDAC3 and REV-ERBα sites

A gene is bound by HDAC3 or REV-ERBα at ZT10, if a predicted binding site is present within 10kb from its TSS. Percentages of genes bound by HDAC3 were compared among the following gene categories: 1. top 10% expressed, middle 10% and bottom 10% expressed genes at ZT10; 2. the up-regulated and down-regulated genes upon loss of HDAC3; 3. the circadian and non-circadian genes. The hypergeometric-based test was used to calculate the p-value of whether or not HDAC3 or REV-ERBα binding is significantly enriched in the gene categories comparing to all known mouse genes. The distribution function for hypergeometric distribution (phyper from the statistics language R) is used as: *pvalue = phyper(q,m,n,k,lower.tail=T/F)*, where *m* is the number of all the genes bound by HDAC3 at the promoter region and *n* is the number of genes without HDAC3 binding, out of *k* genes in certain category (e.g. circadian genes) *q* is the number of genes bound by HDAC3. The *lower.tail* is set to *F* while we calculate the pvalue for enrichment or *T* for depletion. Gene ontology analysis is performed on PANTHER website (Thomas et al., 2003).

Analysis of RORα ChIP-seq

Sequencing reads were mapped to the mouse genome (UCSC mm8) using Bowtie (version 0.12.8). Mapped read must have unique alignment with no more than two mismatches in first 27bp. Only uniquely mapped reads were used for peak calling which was performed using FindPeaks algorithm (0.1% FDR) in the HOMER software suite (version 3.15., Heinz et al., 2010). Read density plot was produced using HOMER. Read density heatmap was generated by seqMiner (Ye et al., 2011). ChIP-seq tracks were visualized by Integrated genome viewer (IGV). Motif mining was performed by HOMER using 200bp window size.

Oil Red O staining

5μm frozen sections were prepared from snap-frozen liver tissues, and fixed in 10% buffered formalin for 3 minutes. The sections were then stained in 0.5% Oil Red O in propylene glycerol overnight for lipid and then in hematoxylin for nucleus for 5 seconds. The procedures were performed by the Morphology Core in the Penn Center for Molecular Studies in Digestive and Liver Diseases.

Hepatic triglyceride assay

Liver samples were homogenized in the TissueLyser (Qiagen) with steel beads in tissue lysis buffer (140mM NaCl, 50mM Tris and 1% Triton-X, pH8.0). Triglyceride concentration in the lysates was then quantified using LiquiColor Triglyceride Procedure No. 2100 (Stanbio).

De novo lipogenesis

Mice were injected with deuterated water (20 ul/g body weight) intraperitoneally and euthanized 3 h post injection. ^{2}H-labeled palmitate was extracted from liver and its trimethylsilyl derivative was analyzed using gas chromatography-electron impact ionization mass spectrometry (GC/MS), and normalized to body water enrichment. The percent contribution of newly made palmitate was determined using the equation: % newly made palmitate =[total ^{2}H-labeled palmitate/ (^{2}H-labeled body water ×n)] ×100 where n is the number of exchangeable hydrogens, assumed to equal 22. The absolute amount of newly synthesized palmitate was determined by multiplying the % newly made palmitate by the concentration of palmitate (Leavens et al., 2009).

Table 2.1 List of Primers for Gene Expression

Gene	Forward Primer	Reverse Primer
Arbp	TCATCCAGCAGGTGTTTGACA	GGCACCGAGGCAACAGTT
Bmal1	TAGGATGTGACCGAGGGAAG	TCAAACAAGCTCTGGCCAAT
Npas2	CAGCTTGAACCCAAAGGAAT	CCATTACAGGAGGGGCTAGG
RORα	TGTTTGATTGATCGGACCAG	CTTGGACATCCGACCAAACT

Table 2.2 List of Primers for ChIP-PCR

Gene	Forward Primer	Reverse Primer
Arbp	GAGGTGGCTTTGAACCAGAG	TCTTTGTCTCTGTCTCGGAAAA
Ins	GGACCCACAAGTGGAACAAC	GTGCAGCACTGATCCACAAT
Bmal1	AGCGGATTGGTCGGAAAGT	ACCTCCGTCCCTGACCTACT
Npas2	TTGCAGAAGCTTGGGAAAAG	TTTCCTGTGGGAGGAGACAG
HDAC3 binding sites		
1	AGACCTGGCAGTTGCATTTT	TGGACGGACAGTCGAGTAGA
2	CCTTGGCTGCATACTGAGGT	AGAGCCAGGTGAGATCCTGA
3	GGGAGGAAATGGAGAAGAGG	CAAATGGAGTTCCCAGCAAT
4	TGGCCAGATGCTCAGAGTTC	ACAGAAGCATGAGGCCAACT
5	AACTCTCAGCCTCCAGAGCA	GGACAGGTCACCAGGGTATG
6	GAGTGTGCGCCAGAGATACA	CCTTGGCTGAGGGACTGTAA
RORα binding sites		
1	TCCTTATGCCACTTCCAAAA	ATGCTAAACCACCCACTGGT
2	GCTGACGTCTACAATTGGGTTA	CAATTGACTCCTTTGGCTCTG
3	TGATGACAGCTTTCCCAACA	GCCCATGTGACATTCCCTAT
4	AGCCAAAACAGGTTGCCTAA	GGTGAAGGTCAGCTGGAAAA
5	CCAAGGTCATGTTCATGCTG	TGGGTCAGTCACTTTGGTCA
6	GTCTGTAATGGGGCCATCG	GGCTGCCAAAACCACTATGT

Chapter 3

A Circadian Rhythm Orchestrated by HDAC3

Controls Hepatic Lipid Metabolism

The text, figures, and legends in this chapter were the work of Dan Feng with the following exceptions. Zheng Sun conducted gene expression microarray, hepatic triglyeride assay, Oil Red O staining and *de novo* lipogenesis assay in the HDAC3$^{fl/fl}$ mice, and provided the picture of livers from the HDAC3$^{fl/fl}$; Alb-Cre mice. Anne Bugge assisted in the hepatic triglyceride assay and Oil Red O staining in the Rev-erbα knockout mice. Tao Liu conducted the bioinformatics analysis. David Zhuo provided technical assistance.

3.1 Abstract

Disruption of the circadian clock exacerbates metabolic diseases including obesity and diabetes. Here we show that histone deacetylase 3 (HDAC3) recruitment to the genome displays a circadian rhythm in mouse liver. Histone acetylation is inversely related to HDAC3 binding, and this rhythm is lost when HDAC3 is absent. Although amounts of HDAC3 levels are constant, its genomic recruitment in liver corresponds to the expression pattern of the circadian nuclear receptor REV-ERBα. REV-ERBα colocalizes with HDAC3 near genes regulating lipid metabolism, and deletion of HDAC3 or REV-ERBα in mouse liver causes hepatic steatosis. Thus, genomic recruitment of HDAC3 by REV-ERBα directs a circadian rhythm of histone acetylation and gene expression required for normal hepatic lipid homeostasis.

3.2 Introduction

3.2a Circadian Metabolism and Histone Acetylation

In mammals, metabolic processes in peripheral organs display robust circadian rhythms, coordinated with the daily cycles of light and nutrient availability (reviewed Green et al., 2008; Ramsey and Bass, 2011). Maintaining the circadian rhythms in metabolism is critical for metabolic homeostasis. Circadian misalignment causes metabolic dysfunction, and people engaged in night-shift work suffer from higher incidences of obesity, diabetes, and metabolic syndrome (De Bacquer et al., 2009; Pietroiusti et al., 2010; Scheer et al., 2009). The molecular basis of this is unknown, but genetic disruption of circadian clock components in mice leads to altered glucose and lipid metabolism (Lamia et al., 2008; Marcheva et al., 2010; Rudic et al., 2004; Turek et al., 2005).

Gene expression profiles in multiple metabolic organs have revealed a circadian control of the transcriptome, which might be mediated by regulation of histone acetylation that alters the structure of the epigenome (Akhtar et al., 2002; Balsalobre et al., 1998; McCarthy et al., 2007; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Zvonic et al., 2006). Histone acetylation undergoes cyclic oscillation with the clock, as evident in mouse liver, not only at the promoters of the clock genes and but also in a genome-wide scale (Curtis et al., 2004; Etchegaray et al., 2003; Naruse et al., 2004; Ripperger and Schibler, 2006). Histone acetylation is complex, involving multiple histone acetyltransferase (HATs) and histone deacetylases (HDACs) (Strahl and Allis, 2000). .

3.2b Histone Acetylation and Histone Deacetylase

In eukaryotes, genomic DNA is packaged with histone proteins. There are four core histone proteins: H2A, H2B, H3 and H4. Histones form an octamer with two copies of each core histone, and each octamer is wrapped by genomic DNA of approximately 146bp, forming the basic unit of chromatin, known as the nucleosome. All histone proteins have a 15-30 amino acid unstructured N-terminal, known as the histone tails. The tails contains multiple lysine residues, which are subject to covalent modification, such as acetylation, methylation and ubiquitination. Here we only discuss histone acetylation.

Numerous evidences demonstrate that histone hyperacetylation is highly correlated with transcription activation, and suggest that histone acetylation can promote transcription. However, the cause-and-effect relationship has not been confirmed (Kimura et al., 2005). Multiple lysines in the histone octamer can be acetylated *in vivo*, including histone H3 lysine 4 (H3K4), H3K9, H3K14, H3K18, H3K23, H3K27, H4K5, H4K8, H4K12 and H4K16. It was originally believed that histone acetylation activates transcription through neutralizing positive charges and reducing the electrostatic interaction between histones and the negatively charged DNA. Such a model was strongly challenged by the findings that acetylation on different lysines has specific effects and that HATs and HDACs have residue specificity (reviewed in Kimura et al., 2005; Strahl and Allis, 2000).

Histone deacetylases (HDACs) can be divided into four different classes: Class I (HDAC1/2/3/8), Class II (HDAC4/5/6/7/9/10), Class III (sirtulins), and Class IV (HDAC11) (reviewed in Witt et al., 2009). Class I HDACs are usually members of the multi-protein corepressor complexes, and are critical for transcriptional regulation and histone modification. For instance, HDAC1 and HDAC2 are in the NuRD complex, while HDAC3 is in the NCoR and SMRT complexes. Here we only focus on HDAC3, which is known to function in the regulation of circadian rhythm and glucose metabolism (Alenghat et al., 2008).

HDAC3 is ubiquitously expressed, and plays an crucial role in development and metabolic homeostasis. Germline deletion of HDAC3 causes embryonic lethality before day 9.5. In mouse embryonic fibroblasts (MEFs), loss of HDAC3 also leads to a delay in

cell cycle progression, cell cycle dependent DNA damage and apoptosis (Bhaskara et al., 2008). Liver-specific knockout of HDAC3 causes hepatic steatosis (Knutson et al., 2008).

3.3 Results

3.3a Circadian Binding of HDAC3 at ZT10 and ZT22 in Liver

We hypothesized that HDAC3 displays different genomic binding and transcriptional activity at different times of the day. To test this hypothesis, we kept wildtype C57Bl/6 mice under 12h-light/12h-dark cycles for at least two weeks in an isolated chamber without disturbance (Figure 3.1). We profiled HDAC3 bindings at two time points: 5pm/ZT10 and 5am/ZT22 using chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq), and discovered diurnal recruitment of HDAC3 to the mouse liver genome. At ZT10, in the light period when mice are inactive, HDAC3 bound over 14,000 sites in adult mouse liver (the HDAC3 ZT10 cistrome). At ZT22, in the dark period when mice are active and feeding, only 120 specific peaks remained (Figure 3.2A). The binding signals at the binding sites decreased significantly from ZT10 to ZT22 (Figure 3.2B, C), which was also validated with ChIP-PCR using a different antibody (Figure 3.3A). HDAC3 binding was absent when HDAC3 was deleted in liver, suggesting that the antibody used for ChIP-seq is highly specific to HDAC3 (Figure 3.3B). Further analysis of the HDAC3 ZT10 cistrome revealed that a majority of these binding sites were distant from transcription start sites (TSSs) or present in introns (Figure 3.4).

HDAC3 recruitment oscillates in a 24-hour cycle (Figure 3.5). Such a rhythm was retained in constant darkness (Figure 3.6A), suggesting that it was controlled by the circadian clock. The liver clock is entrained by food intake (Damiola et al., 2000) and, indeed the pattern of HDAC3 enrichment was reversed when food was provided only during the light period (Figure 3.6B), further supporting the conclusion that the rhythm of HDAC3 genomic recruitment was controlled by the liver clock.

3.3b Histone Acetylation Is Inversely Correlated with HDAC3 Binding

Despite its known role in histone deacetylation and transcriptional repression, HDAC3 recruitment has been reported to be associated with high histone acetylation, RNA polymerase II (Pol II) recruitment, and gene expression in human primary T cells (Wang et al., 2009). In mouse liver, HDAC3 recruitment at ZT10 was also enriched around active genes (Figure 3.7A), and many of these display circadian expression patterns (Hughes et al., 2009)(Figure 3.7B). Thus HDAC3 may have an important role in transient regulation of these active genes by the circadian clock.

Consistent with this hypothesis, we observed decreases in acetylation of histone H3 lysine 9 (H3K9) at ZT10 compared with that at ZT22, the inverse of HDAC3 recruitment to these sites (Figure 3.8A,B). We then deleted HDAC3 in mouse liver by tail vein injection of adeno-associated virus expressing cre-recombinase (AAV-Cre) into adult C57Bl/6 mice homozygous for a floxed HDAC3 allele (HDAC$^{fl/fl}$)(Figure 3.3B). Loss of HDAC3 led to H3K9 acetylation at ZT10 comparable that of wild type (WT) mice at

ZT22 (Figure 3.8). Our results suggest that circadian binding of HDAC3 drives rhythmic histone acetylation in the mouse liver genome.

In consistent with its role in histone deacetylation and transcription repression, HDAC3 binding is associated with transcription repression. The majority of genes whose transcripts were increased 1 week after HDAC3 deletion in liver displayed HDAC3 binding within 10 kb of their TSSs in WT mice at ZT10 (Figure 3.7C). Thus, genome-wide diurnal recruitment of HDAC3 directs a rhythm of epigenomic modification and gene expression.

3.3c REV-ERBα Mediates HDAC3 Recruitment

Although HDAC3 recruitment to the genome was diurnal, the abundance of HDAC3 was constant throughout the light/dark cycle (Figure 3.10A). In addition, HDAC3 does not bind genome directly, and is usually present in the NCoR/SMRT corepressor complex, which mediates transcription repression by nuclear receptors (Guenther et al., 2000; Ishizuka and Lazar, 2003; reviewed in Hu and Lazar, 2000). Moreover, HDAC3 enzyme activity requires interaction with nuclear receptor (NR) corepressors (Guenther et al., 2001). Therefore, we hypothesized that HDAC3 is recruited by nuclear receptors in a circadian manner. In consistent with our hypothesis, *De novo* motif analysis of the HDAC3 binding sites revealed the classical motif recognized by a number of NRs (Figure 3.9).

The NR REV-ERBα is a transcriptional repressor that is expressed in a circadian manner, and the abundance of REV-ERBα protein oscillated in phase with HDAC3 recruitment (Preitner et al., 2002) (Figure 3.10A). REV-ERBα is also known to recruit the NCoR corepressor complex and HDAC3 to repress transcription of Bmal1 (Yin and Lazar, 2005). To test the hypothesis that REV-ERBα recruits HDAC3 in a circadian manner, we used ChIP-seq to identify the REV-ERBα binding sites. At ZT10, the REV-ERBα binding sites overlapped with the majority of HDAC3 binding sites (Figure 3.10B). The binding signals decreased significantly in the Rev-erbα knockout liver, suggesting that the signals are specific to REV-ERBα (Figure 3.10C). Similar to HDAC3, a majority of the REV-ERBα binding sites were distant from TSSs or in the introns (Figure 3.11A,B). In consistent with the hypothesis that REV-ERBα recruits HDAC3 to repress its target genes, most of the genes upregulated in liver depleted of REV-ERBα (Kornmann et al., 2007) were bound by HDAC3 as well as REV-ERBα at ZT10 (Figure 3.11C).

Furthermore, REV-ERBα bound the majority of HDAC3 ZT10 sites at ZT10 but not ZT22 (Figure 3.12B). The extent of overlapping between HDAC3 binding and REV-ERBα binding was surprising given that other NRs can interact with corepressors and HDAC3 (Goodson et al., 2005). However, HDAC3 recruitment was indeed diminished at many sites in Rev-erbα knockout mice (Figure 3.13A), consistent with a critical role for REV-ERBα in HDAC3 recruitment. The residual HDAC3 binding at other sites suggests that other factors also contribute to its recruitment. A likely candidate is REV-ERBβ,

whose DNA binding domain is 95% homologous to the REV-ERBα (Renaud et al., 2000; Retnakaran et al., 1994).

REV-ERBα recruits HDAC3 via the nuclear receptor corepressor (NCoR) (Yin and Lazar, 2005; Zamir et al., 1997). The NCoR ZT10 cistrome largely overlapped with the REV-ERBα cistrome and the HDAC3 cistrome (Figure 3.12A). NCoR was recruited to HDAC3 sites with a similar diurnal rhythm (Figure 3.12B). Like HDAC3, NCoR binding at many REV-ERBα binding sites was also attenuated in Rev-erbα knockout mice (Figure 3.13B). In conclusion, our findings support the hypothesis that HDAC3 is recruited by REV-ERBα through NCoR in a circadian manner.

3.4c HDAC3 Regulates Hepatic Lipid Biosynthesis

In order to understand the biological role of the circadian genomic recruitment of HDAC3 in mouse liver, we performed gene ontology study on genes bound by REV-ERBα and HDAC3, and upregulated in livers depleted of HDAC3. We defined genes bound by REV-ERBα as genes that have at least one REV-ERBα binding site within 10kb from the TSSs. This set of genes are most likely the direct targets of HDAC3, and were enriched in genes involved in lipid metabolic processes (Figure 3.14). Therefore, we hypothesized that circadian binding of HDAC3 plays an important role in regulating lipid homeostasis. Indeed, in chow fed mice in which hepatic HDAC3 was deleted for 2 weeks, we observed dramatic increase in neutral lipid by Oil Red O staining (Figure 3.15A). In consistent with this observation, liver triglyceride content was increased by nearly 10

folds (Figure 3.16B). This was consistent with a fatty liver phenotype of mice depleted of hepatic HDAC3 *in utero* [(Knutson et al., 2008)and Figure 3.15C].

REV-ERBα mediates the circadian recruitment of HDAC3. Therefore we hypothesized that loss of Rev-erbα in liver also causes steatosis. Indeed, in chow-fed 9 week old C57Bl/6 mice genetically lacking Rev-erbα, Oil Red O staining of liver was also increased (Figure 3.16A), and hepatic triglyceride content was nearly double that of WT mice (Figure 3.16B). Both the HDAC3 liver-specific knockout mice and the Rev-erbα knockout mice show normal serum transaminase activity, indicating little or no liver damage (data not shown). The relatively modest hepatic steatosis in the Rev-erbα deficient mice likely reflects a role for HDAC3 in mediating effects of other NRs, including REV-ERBβ, whose circadian expression pattern is similar to that of REV-ERBα (Liu et al., 2008a), but could also reflect a compensatory effect of REV-ERBα depletion in other tissues. Nevertheless the finding that depletion of either REV-ERBα or HDAC3 led to a fatty liver phenotype supports the conclusion that circadian REV-ERBα recruitment of HDAC3 to lipid metabolic genes plays a critical physiological role.

In consistent with increase in lipid content, *de novo* lipogenesis in mice lacking hepatic HDAC3 (Figure 3.17A) or in Rev-erbα knockout mice (Figure 3.17B) was significantly elevated. *De novo* lipogenesis rate was assessed through hepatic palmitate synthesis after injection of deuterated water. This supports that HDAC3 and REV-EPBα suppress hepatic lipid biosynthesis, and provides a molecular mechanism underlying the

observation that hepatic lipogenesis in mice follows a diurnal rhythm (Hems et al., 1975) that is anti-phase to REV-ERBα and HDAC3 recruitment to the mouse genome.

3.4 Discussion and Future Directions

Our findings demonstrate the existence of circadian changes in histone acetylation whose dysregulation has the potential to cause major perturbations in normal metabolic function. Each day, low concentrations of REV-ERBα lead to reduced HDAC3 association with the liver genome while the organism is active and feeding, altering the epigenome to permit lipid synthesis and accumulation until abundance of REV-ERBα increases HDAC3 recruitment to liver metabolic genes halts the lipid build-up (Figure 3.18). When either Rev-erbα or HDAC3 is depleted, this cycle does not occur and fatty liver ensues.

Misalignment of fasting/feeding and sleep/wake cycles with endogenous circadian cycles could disrupt the rhythm of HDAC3 association with target genes and contribute to the fatty liver observed in rotating shift workers as well as people with genetic variants of molecular clock genes (Brunt, 2010; Sookoian et al., 2007).

Although our results suggest that HDAC3 is recruited to the mouse liver genome in a circadian manner, and that HDAC3 recruitment at many sites is dependent on REV-ERBα, we cannot rule out that other nuclear receptors also play a role in the diurnal recruitment of HDAC3. Participation of other nuclear receptors is supported by the observation that mice without HDAC3 exhibited a much more severe fatty liver

phenotype than mice without Rev-erbα. Many other nuclear receptors are expressed in a similar rhythm as REV-ERBα, and can mediate diurnal HDAC3 recruitment. Among them is nuclear receptor REV-ERBβ, which has an almost identical DNA binding domain as REV-ERBα. The Rev-erbβ transcript oscillates almost exactly in phase with the Rev-erbα transcript in liver. *In vitro* studies showed that REV-ERBβ recognizes the same motif and regulates some known REV-ERBα target genes. Another candidate nuclear receptor is PPARα, which shares the same expression rhythm as REV-ERBs in liver (Yang et al., 2006). Motif analysis of the HDAC3 ZT10 cistrome also revealed a DR1 nuclear receptor motif known to be bound by PPARα/RXR heterodimer (data not shown). In addition, Glucocorticoid Receptor has been shown to interact with NCoR. GR recognizes a distinct motif and is stably expressed in liver (Yang et al., 2006). However, its genomic binding is regulated by ligands such as corticosterone, and the plasma corticosterone level is controlled by the circadian clock and is higher at ZT10 (Oster et al., 2006). Moreover, glucocorticoid has been shown to drive daily oscillation of metabolic genes in liver (Reddy et al., 2007).

Another question is whether HDAC3 activity is controlled solely by the circadian clock and REV-ERBs. The answer may be 'No'. First, previous work in our lab showed that REV-ERBα is subject to post-translational regulation by GSK3β and ubiquitin E3 ligase ARF-BP/PAM (Yin et al., 2010). This raised the possibility that REV-ERBα can be regulated by environmental cues and adjusts the genomic binding of HDAC3. Secondly, HDAC3 recruitment by REV-ERBα is regulated by heme binding, and heme may serve as sensor for the intracellular metabolic state (Yin et al., 2007). Furthermore, If HDAC3

binding is regulated by other nuclear receptors, such as PPARα and GR, it is likely to respond to fasting/feeding and hormone secretion at the PPARα and GR binding sites. It may be interesting to separate the HDAC3 bindings that are regulated by the circadian clock from those regulated by metabolic signals.

In vitro studies showed that REV-ERBα regulates target gene expression through both active and passive repression. In active repression involving NCoR and HDAC3, REV-ERBα binds either a DR2 element as a homodimer or two monomer sites spaced apart. In passive repression, REV-ERBα binds the monomer sites and competes with transcription activator RORs. Here we reported that REV-ERBα actively represses clock and metabolic genes by recruiting HDAC3 in liver, but motif analysis did not reveal enrichment of the DR2 element, but instead the monomer motif shared by RORs. The lack of DR2 element suggest that in most cases, REV-ERBα binds to two monomer sites spaced apart and collaboratively recruit NCoR and HDAC3 as shown in the Bmal1 promoter (Yin and Lazar, 2005). Indeed, a motif scan performed later showed that approximately 35% of all the REV-ERB binding sites contain at least two half sites and that many REV-ERB binding sites are located in close proximity to another (less than 2kb) (Bugge et al., 2012). The monomer/ROR motif by the motif analysis suggests that REV-ERBα may repress transcription by directly competing with RORs. And we will explore passive repression in the next chapter.
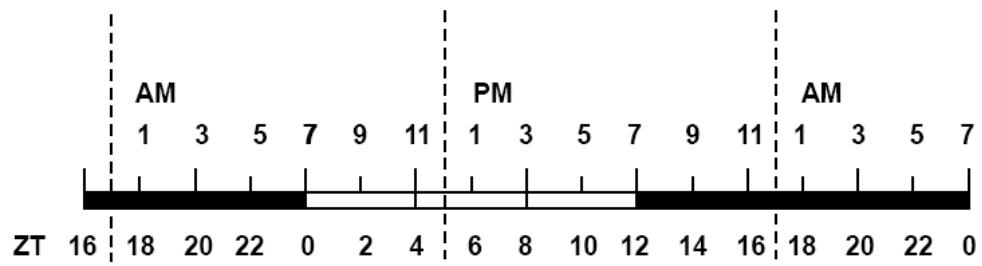
Figure 3.1 Zeitgeber Time. Mice were housed under the regular 12h light/ 12h dark cycles. Light is on at ZT0 (7am), and off at ZT12 (7pm). ZT10 corresponds to 5pm, and ZT22 corresponds to 5am.
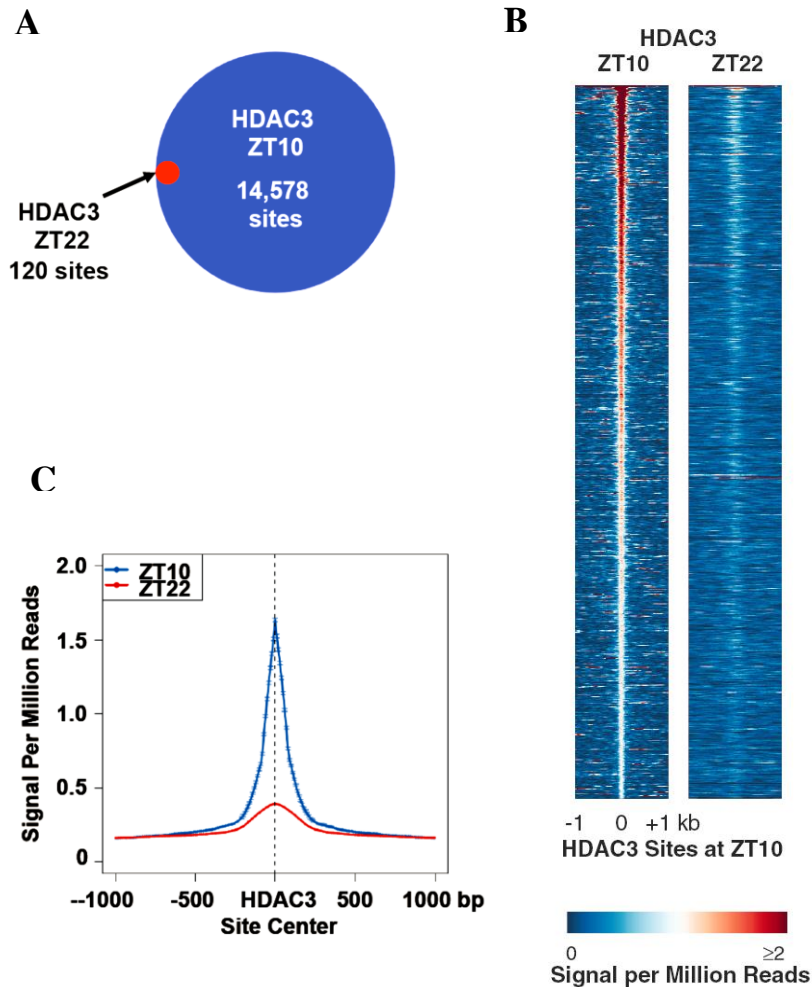
Figure 3.2 Diurnal Binding of HDAC3 in Mouse Liver.

A. Overlapping of HDAC3 cistromes at ZT10 (Blue) and ZT22 (Red). A pair of ZT10 and ZT22 binding sites was considered overlapping when their centers were within 200bp of each other.

B. Heatmap of HDAC3 binding signal at ZT10 (left) and ZT22 (right) from -1kb to +1kb surrounding the center of all the HDAC3 ZT10 binding sites, ordered by strength of HDAC3 binding. Each line represents a single HDAC3 binding site and the color scale indicates the HDAC3 signal (reads encompassing each locus per million total reads).

C. Average HDAC3 signal from -1kb to +1kb surrounding the center of all the HDAC3 ZT10 binding sites. The Y axis represents the HDAC3 signal per million total reads. ZT, Zeitgeber time.
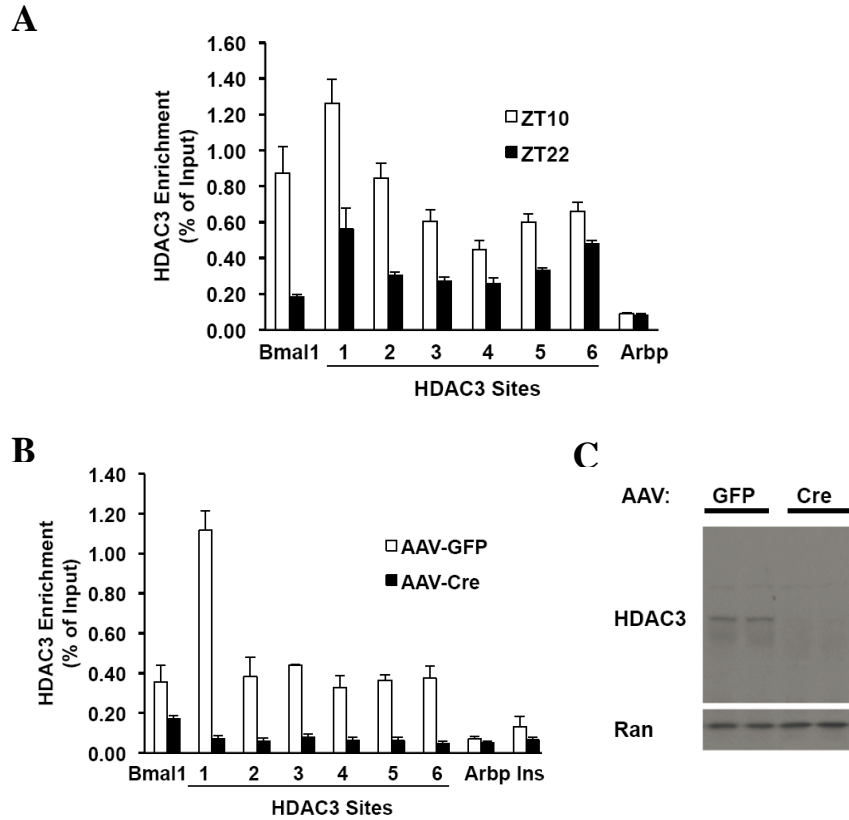
Figure 3.3 Validation of HDAC3 Binding Sites.

A. Diurnal recruitment of HDAC3 as shown by ChIP-PCR with a different HDAC3 antibody (sc-11417). Six HDAC3 binding sites were interrogated by ChIP-PCR. The Bmal1 gene promoter was used as a positive control, and regions close to the TSSs of the Arbp and Ins genes served as negative controls. Values are mean ± s.e.m. (n=4-5).

B. HDAC3 enrichment at HDAC3 binding sites interrogated by ChIP-PCR is largely diminished upon loss of HDAC3.

C. Depletion of HDAC3 in liver from HDAC3^{fl/fl} mice 1 week after injection of the AAV-Cre virus. AAV-GFP virus was used as a negative control and protein levels of HDAC3 and Ran (loading control) were measured by western blot.

A

B
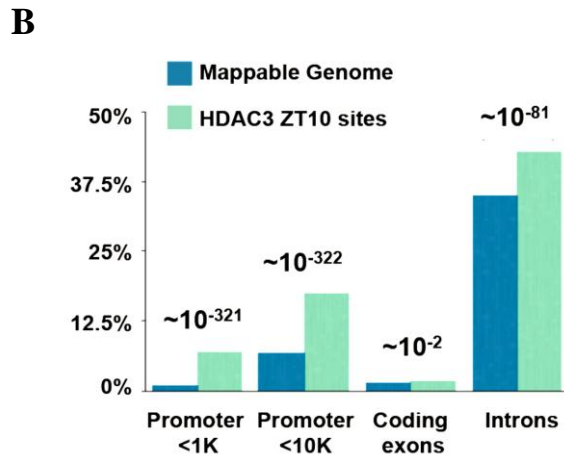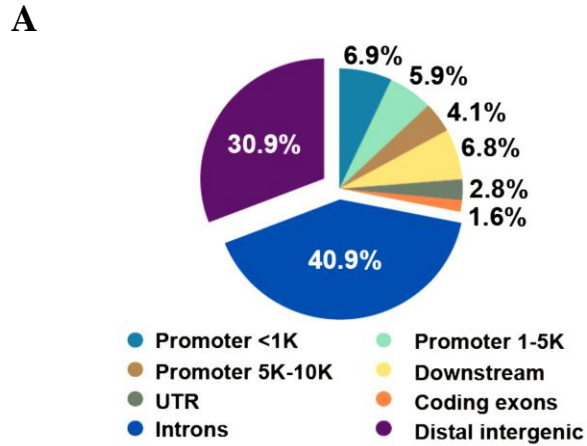
Figure 3.4 Genomic Distribution of HDAC3 ZT10 Cistrome.

A. Distribution of the HDAC3 ZT10 binding sites relative to known genes.

B. Percentage of the HDAC3 ZT10 sites (green) located at different positions relative to known genes in comparison with the mappable mouse genome (blue). The percentage was calculated by CEAS as described in Methods, and the p-values indicate the significance of the differences between the HDAC3 ZT10 cistrome and the mappable mouse genome.
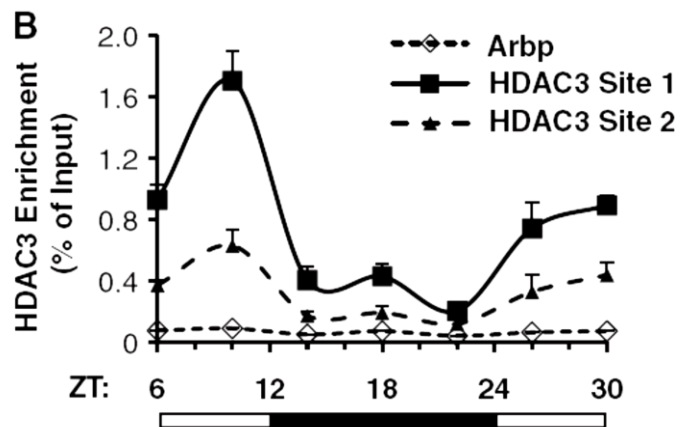
Figure 3.5 HDAC3 Binding Oscillates in a 24h Cycle. HDAC3 recruitment at 2 selected genomic sites over a 24h cycle by ChIP-PCR. Immunoprecipitated DNA was normalized to input. Values are mean ± s.e.m. (n=4-5).
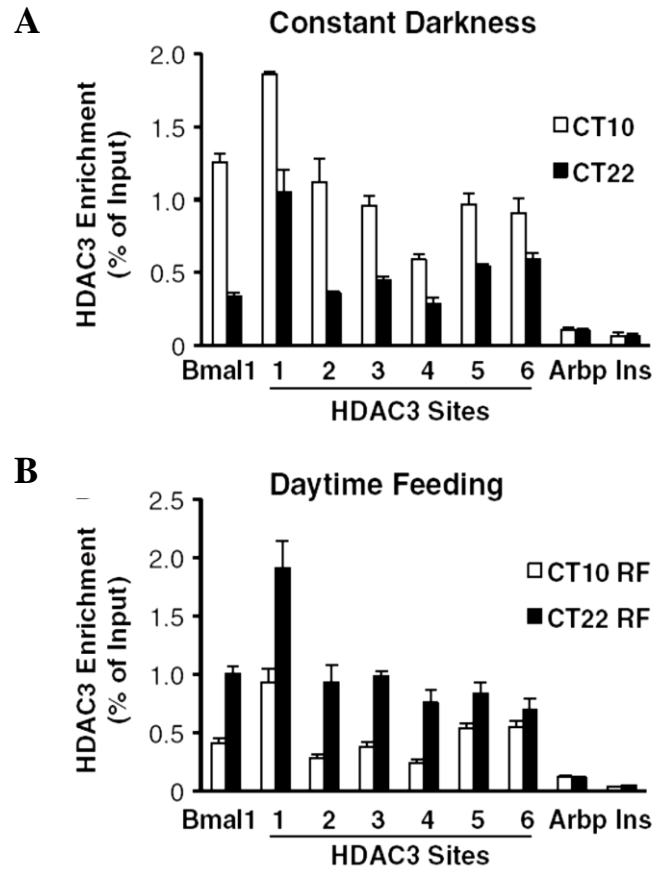
Figure 3.6 Diurnal Binding of HDAC3 Is Controlled by the Liver Clock.

A. Diurnal HDAC3 genomic recruitment is maintained in constant darkness. Six HDAC3 binding sites were assessed by ChIP-PCR (n=4-5). The Bmal1 promoter (Bmal1) served as a positive control, and regions close to the TSS of the Arbp and Ins genes served as negative controls.

B. The rhythm of HDAC3 recruitment is reversed by day-time feeding (n=4-5). RF, food was provided only from ZT3 to ZT11 every day for 2 weeks.
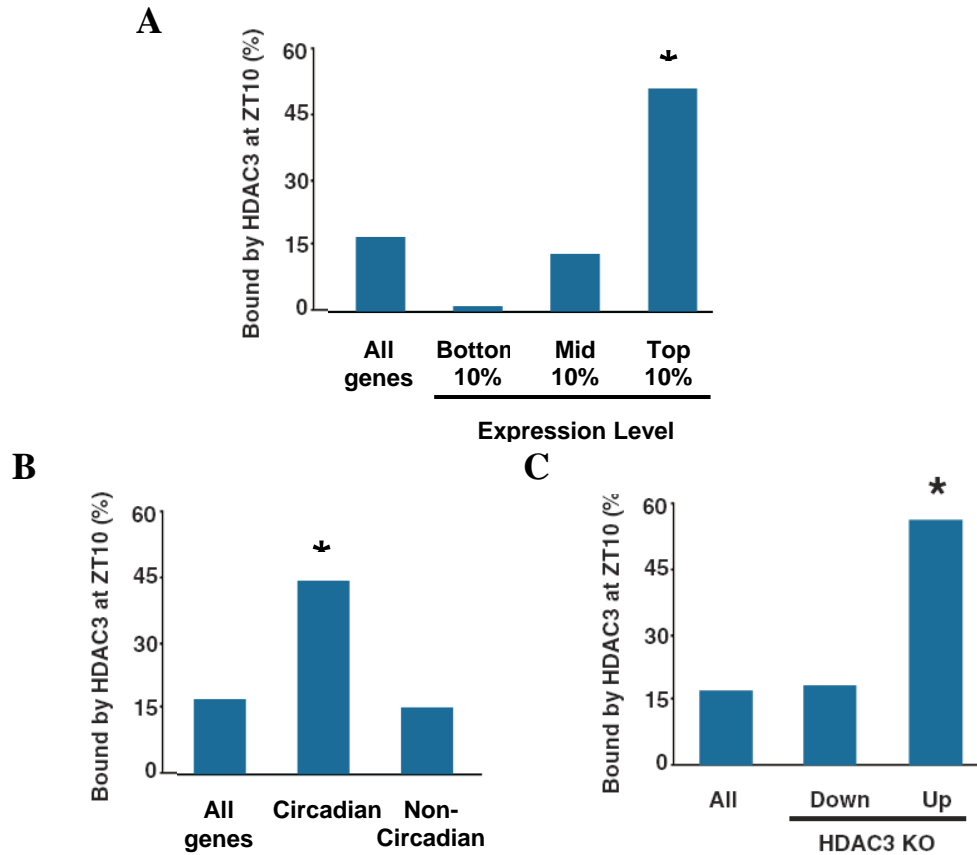
Figure 3.7 HDAC3 Functions as a Transcription Repressor near Active and Circadian Genes.

A. HDAC3 binds mainly to active genes. All mouse RefSeq genes were ranked based on their expression index in ZT10 mouse liver, and genes among the top 10%, middle 10% and bottom 10% were selected. *p-value~10^{-279}

B. HDAC3 binds mainly to circadian genes. All known genes were divided into circadian genes and non-circadian genes. The percentage of HDAC3 bound genes in each category was calculated, and compared with all genes. *p-value~10^{-136}.

C. Genes up-regulated in liver depleted of HDAC3 are significantly enriched for HDAC3 binding at ZT10. Expression arrays of WT and HDAC3 KO liver were performed and analyzed as described in Methods, and the percentage of HDAC3 bound genes in each

category was calculated. *p~10^{-156}. p-values were calculated based on hypergeometic distribution.
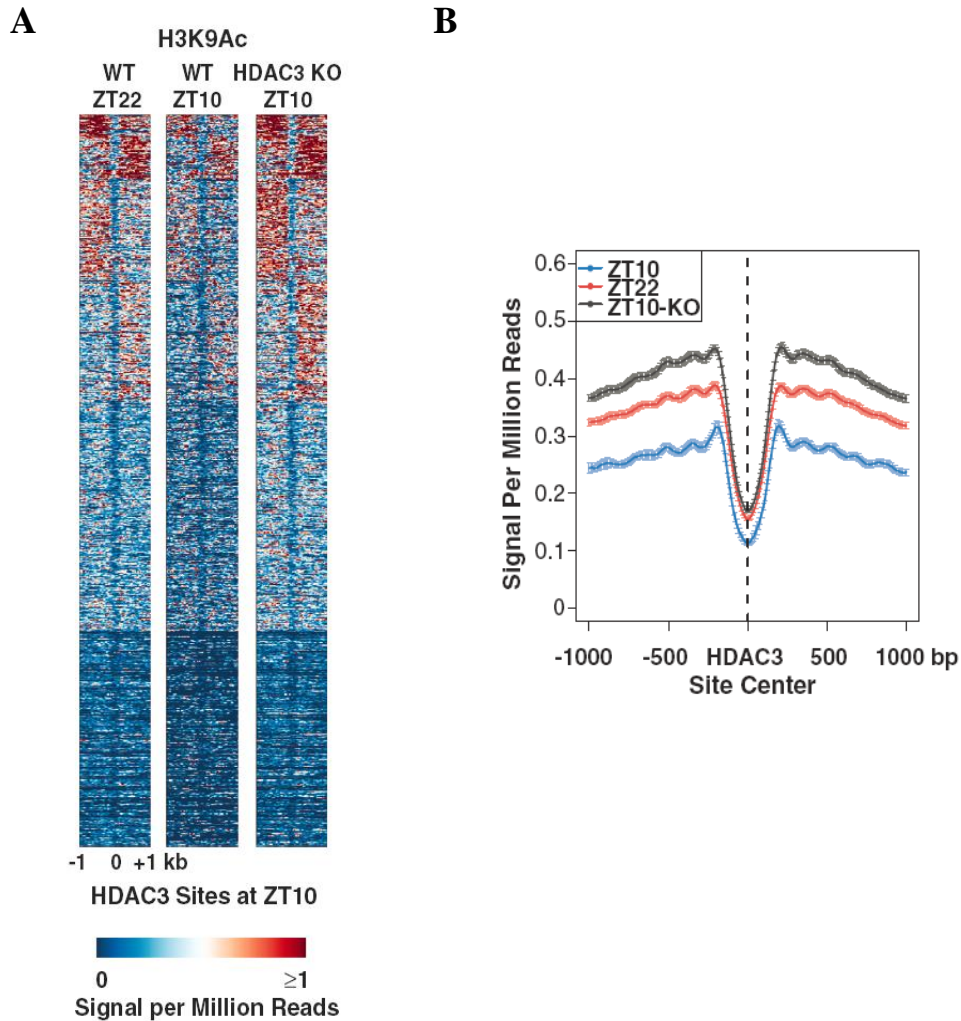
Figure 3.8 Histone Acetylation Is Inversely Correlated with HDAC3 Binding.

A. Heatmap of the histone H3 lysine 9 acetylation (H3K9Ac) signal at ZT22 (left), ZT10 (middle) and ZT10 in liver depleted of HDAC3 (KO) (right) from -1kb to +1kb surrounding the center of all the HDAC3 ZT10 binding sites. Each line represents a single HDAC3 binding site and the color scale indicates the H3K9Ac signal per million total reads. HDAC3 depleted liver was removed from HDAC3^{fl/fl} mice 1 week after injection of AAV-Cre.

B. Average H3K9Ac signal from -1kb to +1kb surrounding the center of all the HDAC3 ZT10 binding sites. The Y axis represents the HDAC3 signal per million total reads.
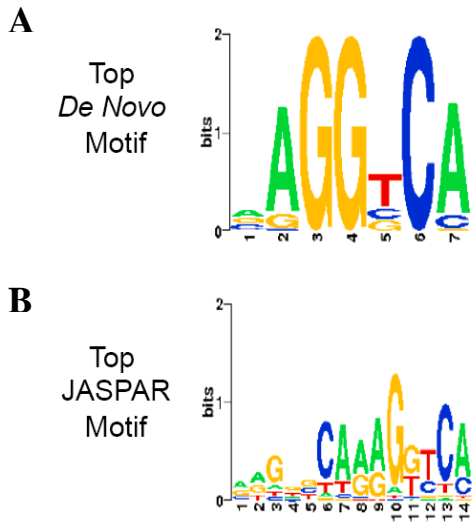
Figure 3.9 Motif Analysis of the HDAC3 ZT10 Cistrome Reveals a Canonical Nuclear Receptor Half Site. Sequences within ± 300bp from the centers of all the HDAC3 sites were used for *de novo* motif analysis (Top) and motif scan against the JASPAR database (Bottom), and the most enriched motif for each analysis is shown.
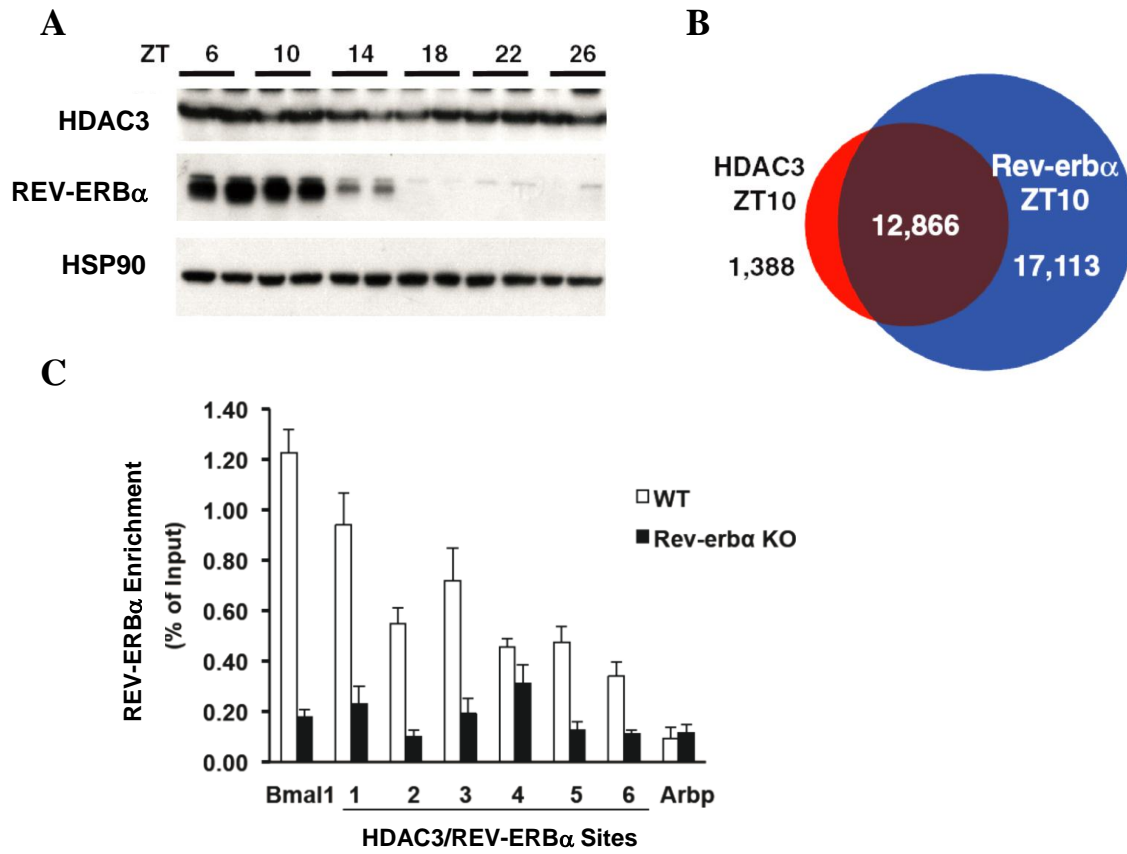
Figure 3.10 REV-ERBa Cistrome Overlaps with HDAC3 Cistrome at ZT10.

A. Immunoblot of HDAC3 and REV-ERBα over a 24h cycle in mouse liver. HSP90 protein levels are shown as loading control.

B. The HDAC3 ZT10cistrome largely overlaps with the REV-ERBα ZT10 cistrome. HDAC3 binding sites and REV-ERBα binding sites were considered overlapping when their centers were within 200bp of each other.

C. REV-ERBα enrichment interrogated by ChIP-PCR is largely diminished in Rev-erbα KO liver at ZT10, demonstrating that the REV-ERBα antibody is specific.
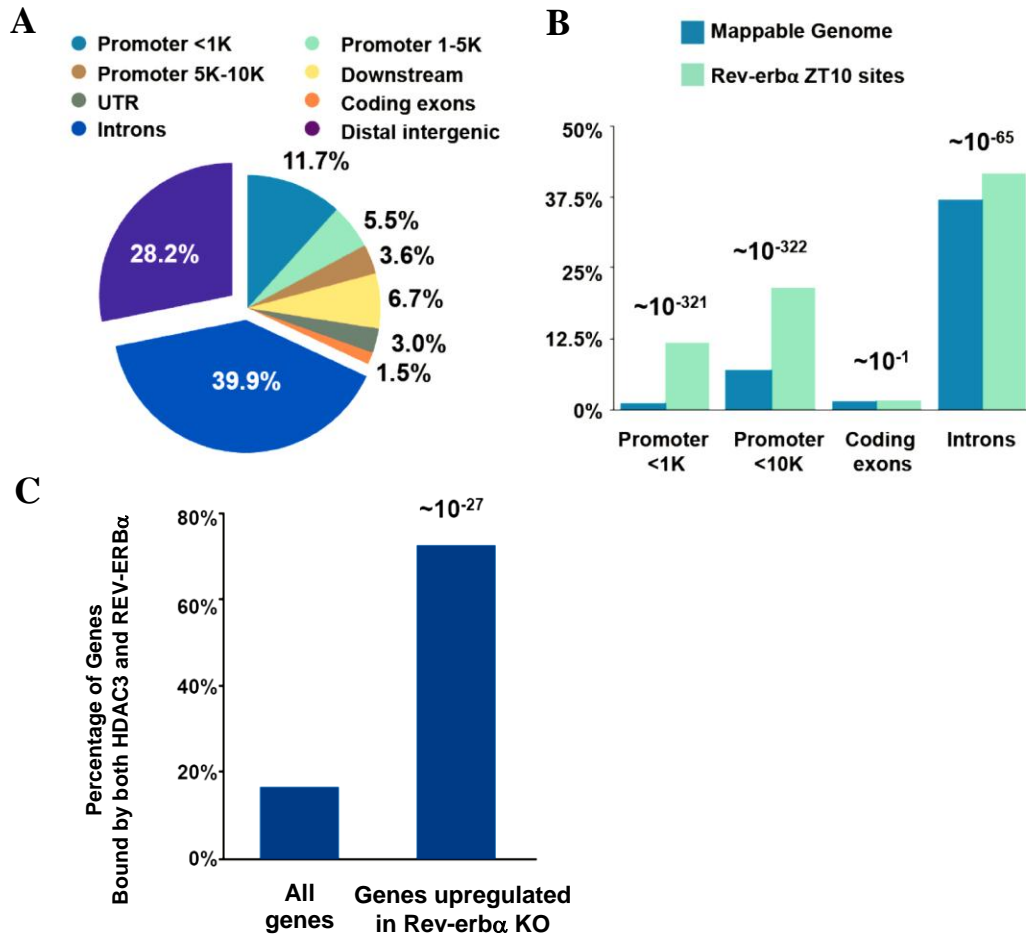
Figure 3.11 Genomic Distribution of the REV-ERB ZT10 Cistrome.

A. Distribution of REV-ERBα ZT10 sites relative to known genes.

B. Percentage of the REV-ERBα ZT10 sites (green) located at different genomic regions relative to known genes compared with the mappable mouse genome (blue). The percentage of all sites in each group was calculated by CEAS.

C. Genes up-regulated in Rev-erbα KO liver are enriched for both HDAC3 and REV-ERBα binding at ZT10 in mouse liver. Genes up-regulated at ZT12 in the liver of the Rev-erbα KO mouse were identified using published expression array data (Le Martelot et al., 2009). Among these, the percentage of genes bound by both HDAC3 and REV-

ERBα were calculated, and compared with all genes. p-values above each column indicates the significance of difference and were calculated based on hypergeometric distribution.
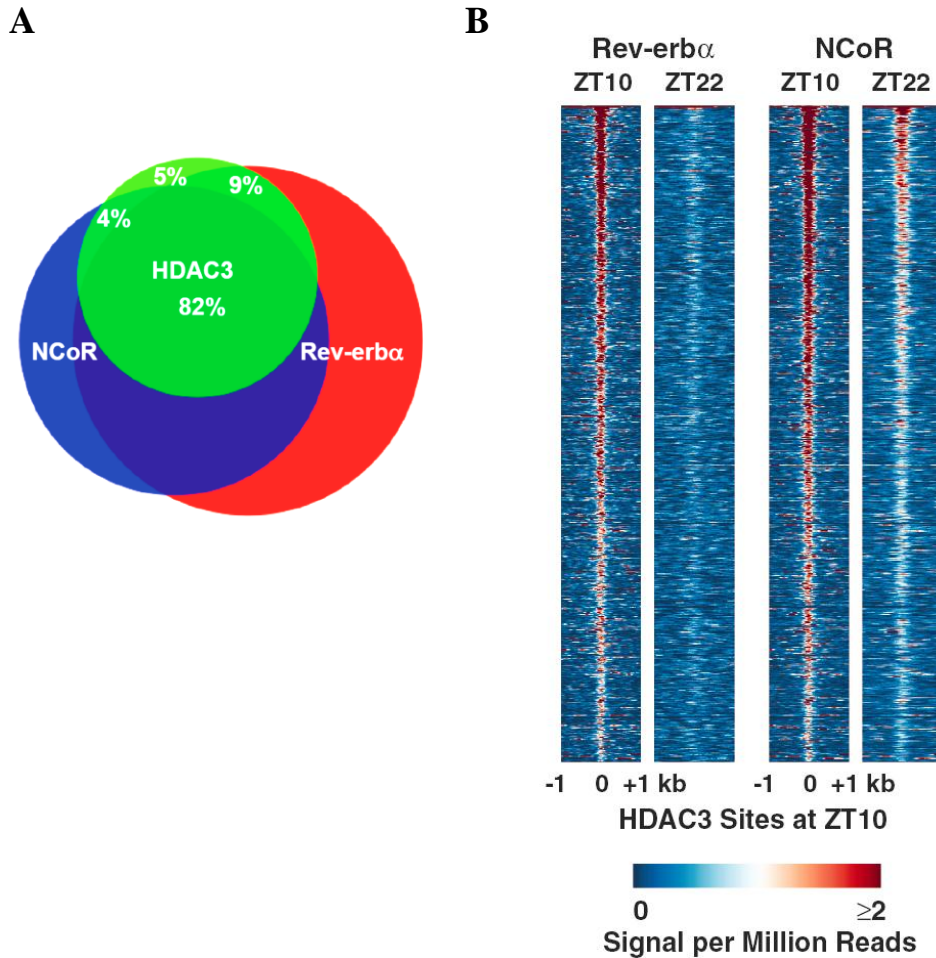
Figure 3.12 REV-ERBα Recruits NCoR Corepressor and HDAC3 with a Circadian Rhythm.

A. HDAC3 (green) ZT10 cistrome largely overlapped with both REV-ERBα (red) and NCoR cistrome at ZT10 (blue). Binding sites were considered overlapping when their centers were within 200bp of each other. Numbers indicates the percentage of all HDAC3 binding sites in each group.

B. Heatmap of REV-ERBα binding at ZT10 and ZT22 (left) and of NCoR at ZT10 and ZT22 (right), both at HDAC3 ZT10 sites ordered as in Figure 3.2. Each line represents a single HDAC3/REV-ERBα binding site and the color scale indicates the signal per million total reads.
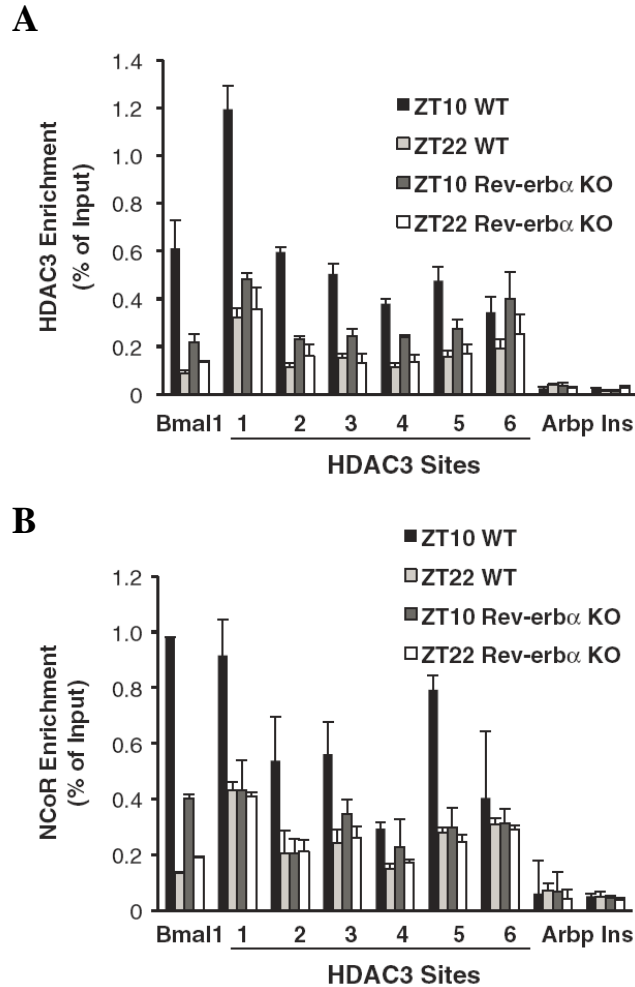
Figure 3.13 Loss of HDAC3 and NCoR Recruitment upon Rev-erbα Deletion.

A. HDAC3 recruitment to six binding sites in liver from mice lacking Rev-erbα was interrogated by ChIP-PCR.

B. NCoR recruitment to six binding sites in liver from mice lacking Rev-erbα was interrogated by ChIP-PCR. The Bmal1 gene promoter (Bmal1) was used as a positive control, and regions close to the TSS of the Arbp and Ins genes served as negative controls. Values are mean ± s.e.m. (n=3).

Genes bound by HDAC3 and Rev-erbα and up-regulated in HDAC3 KO

Biological Processes	p-value
Lipid metabolic process	1.72E-24
Metabolic process	8.22E-21
Cellular amino acid and derivative metabolic process	8.33E-19
Primary metabolic process	8.30E-17
Coenzyme metabolic process	1.84E-14
Carbohydrate metabolic process	1.78E-11

Figure 3.14 HDAC3 and REV-ERBα Regulate Lipid Metabolic Genes. Gene ontology analysis of the HDAC3 and REV-ERBα-bound genes that were up-regulated in liver depleted of HDAC3 was performed using Panther (Thomas et al., 2003).
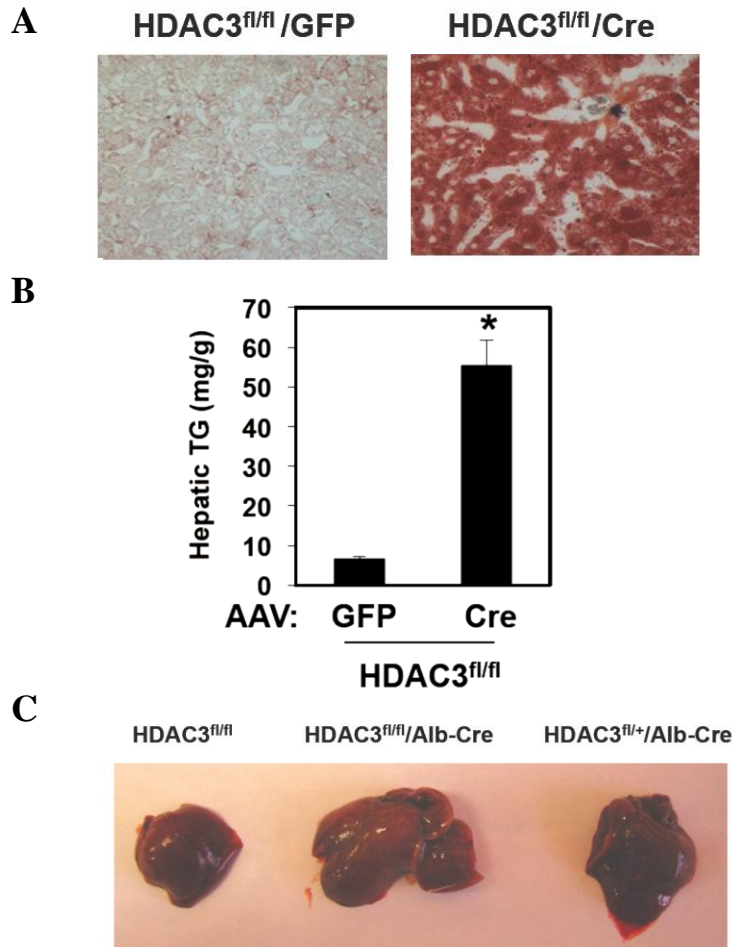
Figure 3.15 Loss of HDAC3 Causes Liver Steatosis.

A. Oil Red O staining of liver from 14 week old HDAC3$^{fl/fl}$ mice 2 weeks after tail vein injection of AAV-GFP or AAV-Cre.

B. Hepatic triglyceride (TG) levels in mice treated as in "A". Values are mean ± s.e.m. (n=4). *p<0.05 by student's t-test.

C. Enlarged and fatty liver in 8 week old male HDAC3$^{fl/fl}$/Alb-Cre mice. Livers from HDAC3$^{fl/fl}$ mice and HDAC3$^{fl/+}$/Alb-Cre mice are used as negative controls. Alb-Cre, the cre recombinase gene under a liver-specific Albumin promoter.

A

B
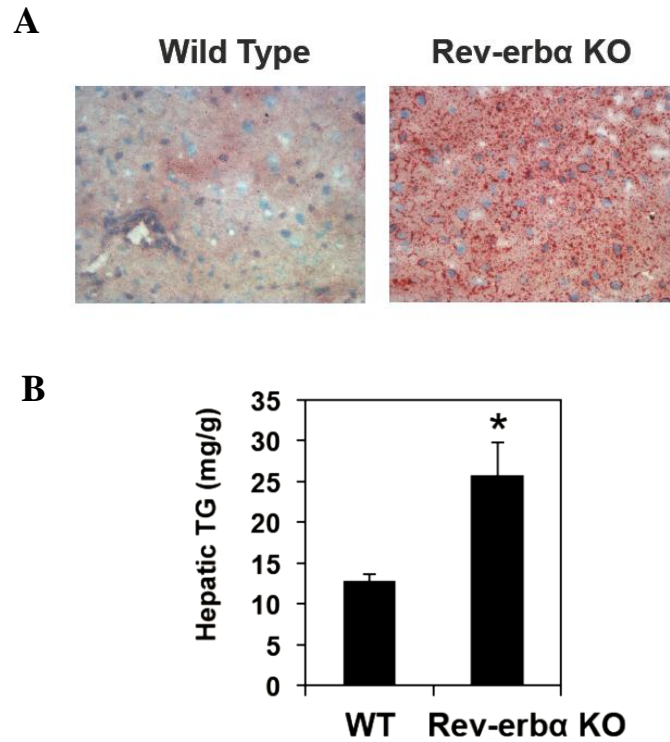
Figure 3.16 Loss of Rev-erbα Causes Hepatic Steatosis.

A. Oil Red O staining of liver from 9 week old WT and mice lacking Rev-erbα (Rev-erbα KO).

B. Hepatic TG levels in from livers from 9 week old WT and mice lacking Rev-erbα (Rev-erbα KO). Values are mean ± s.e.m. (n=4). *$p<0.05$ by student's t-test.
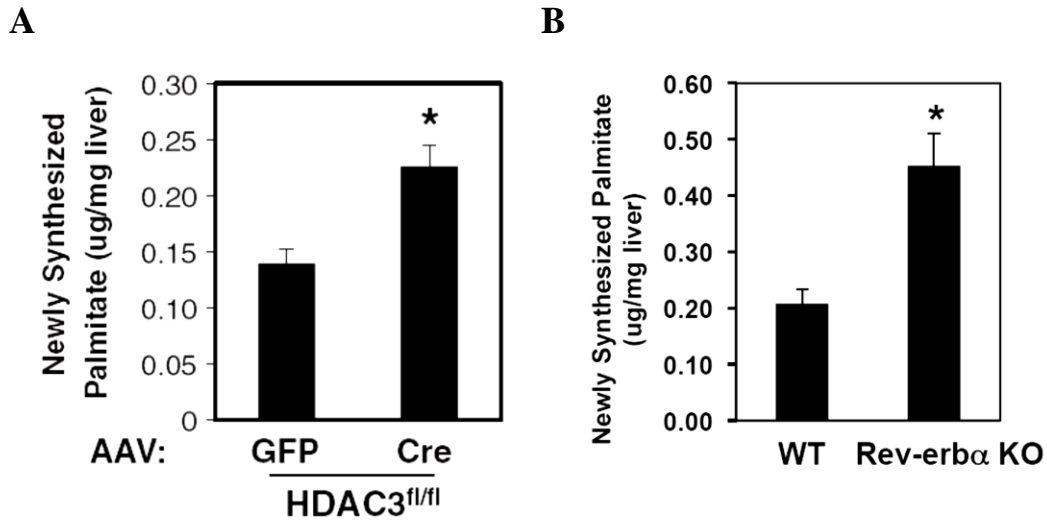
Figure 3.17 Hepatic Lipogenesis Is Upregulated in the Absence of HDAC3 or REV-ERBα.

A. Hepatic *de novo* lipogenesis (DNL) in 13 week old HDAC3$^{fl/fl}$ mice 1 week after infection with AAV-Cre or with AAV-GFP. Values are mean ± s.e.m. (n=7-8).

B. Hepatic *de novo* lipogenesis (DNL) in 14 week old Rev-erbα KO mice or WT mice. Hepatic DNL was measured as newly synthesized and ^{2}H labeled palmitate as in Methods. Values are mean ± s.e.m. (n=7-8). *p<0.05 by student's t-test.
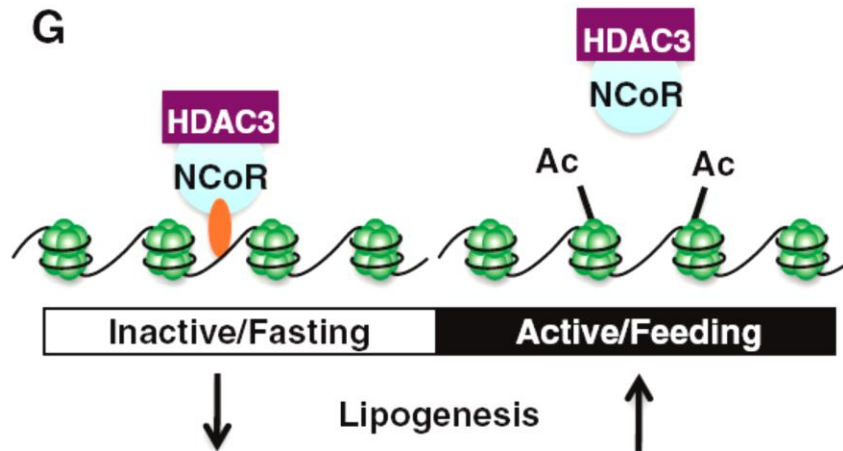
Figure 3.18 Model of REV-ERBα and HDAC3 Function in Liver. In the day time, when mice are inactive and fasting, REV-ERBα recruits NCoR/HDAC3, which deacetylates histone and represses lipid biosynthetic genes. At night, when mice are active and feeding, REV-ERBα is absent, and the dissociation of NCoR/HDAC3 from the genome allows histone acetylation and transcriptional activation of lipid biosynthetic genes. Through this mechanism, REV-ERBα and HDAC3 contribute to the circadian oscillation of lipogenesis in liver.

Table 3.1 List of ChIP-seq Data

	total reads	Non-redundant	# of peaks (SPMR>=1)
Rev-Erbα 5pm (2 reps)	30,637,929	19,995,375	30,787
Rev-Erbα 5am	12,782,094	11,451,185	984
HDAC3 5pm (3 reps)	42,315,338	28,873,243	14,578
HDAC3 5am (2 reps)	34,786,996	33,139,525	120
NCoR 5pm	18,875,895	18,236,535	23,817
NCoR 5am	16,833,313	16,286,483	5,258
H3K9Ac 5pm	12,022,705	NA	122,331 (NPS)
H3K9Ac 5pm after HDAC3 KO	19,904,250	NA	257,421 (NPS)
H3K9Ac 5am	22,073,824	NA	224,639 (NPS)
5pm Input	20,346,220	16,564,474	NA
5am Input	24,056,755	15,555,656	NA

Chapter 4

Circadian Regulation of Hepatic Metabolism

by REV-ERBβ and RORα

The text, figures, and legends in this chapter were the work of Dan Feng with the following exceptions. Experiments on REV-ERBβ from Figure 4.2 to 4.5 were jointly conducted by Dan Feng and Anne Bugge, and the figures were plotted by Anne Bugge except for Figure 4.3B. Experiments from the *Genes & Development* manuscript that were conducted solely by Anne Bugge were not included. Bin Fang conducted the bioinformatics analysis on RORα ChIP-seq. David Steger provided GR ChIP-seq data in liver. Zheng Sun provided livers from the HDAC3$^{fl/fl}$ mice. Lindsey Peed provided technical assistance.

4.1 Abstract

The nuclear receptor REV-ERBα regulates circadian rhythm and metabolism. In particular, REV-ERBα, through HDAC3, directs a circadian rhythm of histone acetylation and gene expression that is crucial for metabolic homeostasis. But Rev-erbα deletion has modest effects on both circadian rhythms and metabolism, and it has been considered to be a secondary regulator of the cell-autonomous clock. Here we report that a highly related nuclear receptor REV-ERBβ binds genome with a circadian rhythm similar to that of REV-ERBα. Depletion of REV-ERBβ in Rev-erbα knockout liver further decreases HDAC3 recruitment, de-represses clock genes, and exacerbates hepatic steatosis. Loss of both REV-ERBs completely abolishes the circadian rhythm, as shown in mouse embryonic fibroblasts. These findings establish the two REV-ERBs as major regulators of both clock function and metabolism. In addition to HDAC3, REV-ERBs can passively repress gene expression by competing with RORs. We report that RORα binds Bmal1 and Npas2 genes with a diurnal rhythm anti-phase to that of REV-ERBs. REV-ERBs represses RORα binding at ZT10 and thus drives the diurnal recruitment of RORα, which is expressed at a constant level. Repression by REV-ERBs is independent of HDAC3, suggesting that REV-ERBs represses RORα binding through direct competition. We identify thousands of competing sites similar to those in Bmal1 and Npas2 genes by RORα ChIP-seq at ZT10 and ZT22, many of which are in close proximity of clock and metabolic genes. Functions of RORα in liver will be tested in mouse models of liver-specific deletion of RORα and its paralog RORγ. We also discover thousands of RORα binding sites with little or no difference in RORα binding between ZT10 and ZT22, and

sites where RORα binds in phase with REV-ERBs. How RORα recruitment is regulated at these non-competing or in-phase sites remains to be elucidated.

4.2 Introduction

4.2a REV-ERBβ

In Chapter 3, we show a circadian rhythm of REV-ERBα binding at thousands of genomic sites, which is consistent with the dramatic oscillation of REV-ERBα protein. We also show that REV-ERBα recruits the NCoR corepressor complex and HDAC3, and drives the circadian oscillation of histone acetylation and gene expression. We discovered that REV-ERBα and HDAC3 binding are enriched near lipid metabolic genes, and that loss of Rev-erbα or HDAC3 leads to hepatic steatosis. However, loss of Rev-erbα has minor effects in comparison with loss of HDAC3, raising the question whether other nuclear receptors also play a role in recruiting HDAC3 and suppressing hepatic lipid biosynthesis.

Loss of Rev-erbα also has a modest effect on circadian rhythm (Preitner et al., 2002). Although the circadian expression of Bmal1 and Clock is severely blunted in the liver of the Rev-erbα-deficient mice, the circadian expression of Cry2 and Per2 are almost intact. When these mice were put into constant darkness, they maintained circadian locomoter activity, suggesting that the circadian rhythm was not disrupted, though the circadian period is slightly yet significantly shorter in these mice (Preitner et al., 2002). These findings argue that REV-ERBα only plays an auxiliary role in the circadian clock.

However, the basic clock machinery is featured by the redundancy of its components (DeBruyne et al., 2007; van der Horst et al., 1999; Shi et al., 2010; Zheng et al., 2001). Therefore, we hypothesized that a closely related nuclear receptor REV-ERBβ can compensate for loss of REV-ERBα, and the REV-ERBs are essential components of the core clock.

Like REV-ERBα, REV-ERBβ is also considered an orphan nuclear receptor (Retnakaran et al., 1994). It is the most closely related nuclear receptor to REV-ERBα (>95% identical in the DNA binding domain, and >65% identical in the ligand binding domain). Only estrogen receptors (ERs) have a similar high degree of conservation at the DNA binding domain among the two subtypes. The REV-ERBs differ in the N-terminal A/B domain and the hinge region. REV-ERBβ also recognizes the canonical REV-ERBα motif, which is an AGGTCA half site preceded by an A/T-rich sequence at the 5' (Dumas et al., 1994; Pardee et al., 2009; Woo et al., 2007) (Figure 4.1). Rev-erbβ is also ubiquitously expressed, and has been shown to regulate Bmal1 and ApoC-III, the known targets of REV-ERBα (Liu et al., 2008a; Wang et al., 2007, 2008), as well as genes involved in lipid absorption in skeletal muscle cells (Ramakrishnan et al., 2005). Like REV-ERBα, REV-ERBβ also binds heme, which enhances corepressor recruitment and transcriptional repression (Pardee et al., 2009; Raghuram et al., 2007). Through heme, REV-ERBs can sense the redox state and gas such as O_2, NO and CO (Pardee et al., 2009). In addition, heme binding can be regulated by the redox state of the REV-ERBβ protein (Gupta and Ragsdale, 2011). Other than the above findings, little is known about the role of REV-ERBβ in circadian rhythms and metabolism.

4.2b RORs

The ROR subfamily of nuclear receptors consists of three members: RORα (NR1F1), RORβ (NR1F2) and RORγ (NR1F3). They are classified as orphan receptors, and their endogenous ligands are yet to be discovered. Each ROR gene generates several different transcripts by using different promoters and/or through alternative splicing, and produces isoforms that differ in the N-terminal protein. Each ROR gene and isoform has its own distinct profile of tissue-specific expression (reviewed in Jetten, 2009). For instance, RORα4 and RORγ1 are expressed in liver, and RORγ2 (RORγt) is expressed specifically in T cells (Chauvet et al., 2002; He et al., 1998).

RORs have a typically nuclear receptor structure that contains an N-terminal A/B domain, a DNA binding domain, a hinge region and a ligand binding domain. It binds as a monomer to the ROR response element, which consists of an AGGTCA nuclear receptor half site preceded by an A/T-rich sequence (Carlberg et al., 1994; Forman et al., 1994; Giguère et al., 1994; Medvedev et al., 1996). The DNA binding domain of RORs is 65% homologous to that of REV-ERBs, the highest among all nuclear receptor subfamily (Forman et al., 1994). In consistent with the high homology, RORs have been shown to compete with REV-ERB binding *in vitro* (Forman et al., 1994), and to regulate the clock genes including Bmal1 and Npas2 through the same binding sites as REV-ERBs (Akashi and Takumi, 2005; Crumbley et al., 2010; Guillaumond et al., 2005; Takeda et al., 2011) (Figure 4.1).

Unlike REV-ERBs, RORs have Helix 12, which allows them to interact with coactivators, including SRC-1, SRC-2, PGC-1a, p300 and CBP (reviewed in Jetten, 2009). Particularly, RORα activates G6pase expression by recruiting SRC-2 and regulates hepatic glucose production (Chopra et al., 2008). RORs also interact with corepressors, including NCoR and SMRT (reviewed in Jetten, 2009). The transcriptional activity of RORs can be regulated by several agonists and antagonists. For instance, 7-oxygenated sterol and 24S-hydroxycholesterol functions as inverse agonists for RORs by inhibiting coactivator recruitment, while a few other hydroxycholesterol functions as agonists by promoting coactivator recruitment (Jin et al., 2010; Wang et al., 2010a, 2010b).

RORs play an important role in a variety of physiological processes, including circadian rhythms, metabolism and inflammation (reviewed in Jetten et al., 2013). Genetic ablation of RORα or RORβ causes aberrant circadian behaviors as measured by lomocotor activity (Akashi and Takumi, 2005; André et al., 1998). RORα and RORγ have been shown to regulate Bmal1 and Npas2 expression (Akashi and Takumi, 2005; Crumbley et al., 2010; Guillaumond et al., 2005; Takeda et al., 2011). RORγ1 expression is directly regulated by BMAL1/CLOCK, and exhibits a circadian pattern (Mongrain et al., 2008; Takeda et al., 2012). In metabolism, genetic ablation of RORα downregulates a number of lipid metabolic genes, and prevents age- or diet-induced liver steatosis (Kang et al., 2007; Lau et al., 2008; Raspé et al., 2001). In addition to hepatic lipid metabolism, RORs are also involved in glucose metabolism, insulin sensitivity, and metabolism in adipose tissue and muscle (reviewed in Jetten, 2009; Jetten et al., 2013).

4.3 Results

4.3a REV-ERBβ Protects Circadian Rhythms and Metabolic Homeostasis from Loss of Rev-erbα

We hypothesized that REV-ERBβ protects the circadian clock and metabolic homeostasis in the Rev-erbα deficient mice, which accounts for the modest effects of Rev-erbα deficiency. REV-ERBβ is known to bind the same response elements as REV-ERBα *in vitro*. Previous studies in our lab revealed that 92% of the REV-ERBβ ZT10 cistrome overlapped with the REV-ERBα ZT10 cistrome and that the REV-ERBβ binding signals were highly correlated with that of REV-ERBα (r=0.78) (Bugge et al., 2012). The cistromic study indicated that REV-ERBβ shares many gene targets as REV-ERBα. Gene ontology analysis of genes with at least one shared binding site within 10kb from their TSSs revealed enrichment for metabolic processes, within lipid metabolism among the top three specialized pathways (Bugge et al., 2012).

Indeed REV-ERBβ was expressed with a circadian rhythm similar to the rhythm of REV-ERBα. Rev-erbβ mRNA reached its peak around ZT10/5pm, and reached its trough at between ZT18/1am and ZT2/9am (Figure 4.2A), which was consistent with its protein (Bugge et al., 2012). Its peak lagged slightly behind that of Rev-erbα, which is between ZT6/1pm and ZT10/5pm. The genomic binding of REV-ERBβ was highly correlated with its expression level, as shown in the Bmal1 promoter and the Npas2 promoter (Figure 4.2B). REV-ERBβ showed the strongest binding between ZT10/5pm and ZT14/9pm, while REV-ERBα shows the strongest binding at ZT10/5pm (Figure 4.2C).

The similar diurnal rhythm and the strong correlation of REV-ERBα and REV-ERBβ binding in mouse liver support that Rev-erbβ represses many of the same genes as REV-ERBα at the same time of the day.

To test this hypothesis, we depleted hepatic REV-ERBβ using an adenovirus encoding a shRNA sequence targeting Rev-erbβ (adeno-shRev-erbβ). Rev-erbβ mRNA and protein levels were down-regulated by at least 80% one week after virus injection [Figure 4.3A and (Bugge et al., 2012)]. In Chapter 3, we showed that REV-ERBα recruited the NCoR corepressor complex and HDAC3 to repress target genes, and observed residual NCoR and HDAC3 binding upon loss of Rev-erbα. Here we showed that loss of REV-ERBβ also decreased HDAC3 binding at the Bmal1 promoter by approximately 50%, comparable to that in the Rev-erbα knockout mice (Figure 4.3B). More importantly, deficiency of both REV-ERBs further decreased HDAC3 binding (Figure 4.3B), indicating that upon loss of either REV-ERB, the remaining one mediates the residual recruitment of HDAC3 and continually represses the shared targets. In consistent with the loss of HDAC3 binding, Bmal1 is elevated upon loss of REV-ERBβ, and is further elevated upon loss of both REV-ERBs (Figure 4.3A). At the promoter of Npas2, neither depletion of REV-ERBβ or knockout of Rev-erbα significantly lowered HDAC3 binding (Figure 4.3B), which explained the minimum change in Npas2 expression (Figure 4.3A). However, deficiency of both REV-ERBs downregulated HDAC3 binding by approximately 50% (Figure 4.3B), leading to a dramatic increase in Npas2 expression (Figure 4.3A). In this case, one REV-ERB can almost completely compensate for loss of

the other. These findings may explain the modest effects of Rev-erbα knockout in both circadian rhythms and metabolism.

To test the hypothesis that REV-ERBβ protects the liver from steatosis upon loss of Rev-erbα, we measured the triglyceride content in the REV-ERBα/β depleted liver. One week after injection of adeno-shRev-erbβ, hepatic lipid content in the Rev-erbα knockout liver was further elevated (Figure 4.4A). Increase in hepatic lipid content was also confirmed by Oil Red O staining for neural lipid (Figure 4.4B).

To test the hypothesis that REV-ERBβ protects the circadian rhythm upon loss of Rev-erbα, we isolated mouse embryonic fibroblasts (MEFs) from both wildtype and Rev-erbα knockout mice, and treated them with adeno-shRev-erbβ. Upon serum shock, MEFs with normal REV-ERB levels were synchronized and displayed circadian oscillation of the clock genes, including Bmal1 and Cry1. Oscillation of Bmal1 and Cry1 was maintained when either REV-ERB was depleted, and was completely abolished when both REV-ERBs were depleted, proving that REV-ERBβ is protecting the circadian clock in the absence of REV-ERBα. REV-ERBs are essential components of the core clock machinery.

In conclusion, we discovered that REV-ERBβ displays circadian genomic binding similar to REV-ERBα and that REV-ERBβ shares many genomic binding sites and target genes with REV-ERBα. Moreover, both REV-ERBs repress gene transcription by recruiting HDAC3, and are key regulators of the circadian clock and lipid metabolism. The

redundancy of REV-ERBα and REV-ERBβ protects circadian rhythms and metabolism, and deficiency of both REV-ERBs abolishes circadian rhythms and disrupts metabolic homeostasis.

4.3b REV-ERBs Compete with RORα for Genomic Binding at the Clock Genes

We hypothesized that REV-ERBs repress many clock and metabolic genes by directly competing with RORα for genomic binding adjacent to these genes, and that competition with RORα is crucial for maintaining the circadian rhythm and metabolic homeostasis. We first tested this hypothesis at the promoters of two clock genes, Bmal1 and Npas2. Previous studies already showed that REV-ERBα binds these promoters in a circadian manner, with a peak at ZT10 and a trough at ZT22. RORα bound the Bmal1 promoter and the Npas2 promoter at ZT22, when both REV-ERBs are absent in liver (Figure 4.6A). At ZT10, when REV-ERBs occupy these promoters, RORα binding were largely inhibited (Figure 4.6A). The anti-phase rhythm of ROR binding to these promoters, despite of the constant expression of RORα (Figure 4.6B), supports the idea that REV-ERBs compete with and repress RORα binding. Indeed, RORα binding at the Bmal1 promoter was significantly upregulated when Rev-erbα was deleted genetically or when REV-ERBβ was depleted by injection of adeno-shRev-erbβ, and was dramatically up-regulated in the absence of both Rev-erbs, to a level close to that at ZT22 (Figure 4.7A), but no change was observed in the RORα protein level (data not shown). At the Npas2 promoter, significant upregulation of RORα binding was only observed in the absence of

both Rev-erbs (Figure 4.7A). Changes in RORα binding were consistent with increase in gene expression (Figure 4.3A). Moreover, RORα binding showed no increase when HDAC3 was deleted in the liver and Rev-erbα binding was unaffected (Figure 4.7B), suggesting that REV-ERBs suppress ROR binding through direct competition, independent of chromatin modification (Figure 4.7C).

4.3c Genome-wide Profile of RORα Binding

We hypothesized that the competition between REV-ERBs and RORα is not limited to Bmal1 and Npas2, but is a genome-wide phenomenon. To test this hypothesis, we performed RORα ChIP-seq in mouse liver harvested at ZT10 and ZT22. We performed peak calling using HOMER, and identified approximately 13,600 RORα binding sites at ZT10, and approximately 20,000 RORα binding sites at ZT22 (Figure 4.8A). Almost all of the RORα cistrome (including binding sites at ZT10 and ZT22) was also bound by REV-ERBα (Figure 4.8B). We then normalized the RORα signal to the total number of reads in each dataset, and compared the RORα signal at ZT10 and ZT22 (Figure 4.9A). We successfully identified 4740 competing sites where RORα binding increases by at least 1.5 fold from ZT10 to ZT22. Many of the competing sites located near clock genes including Bmal1, Npas2 and Cry1, metabolic genes including G6pase, Elovl5, Gpam and Hmgcs2, and metabolic regulators including PGC-1β, E4bp4 and Insig2 (data not shown). The competing sites are only a small fraction of the RORα cistrome. We also identified 7520 non-competing sites (less than 1.2 fold difference between ZT10 and ZT22) and 2486 in-phase sites (at least 1.5 fold decrease from ZT10 to ZT22). Among the

competing sites are the Bmal1 promoter (Figure 4.9B) and the Npas2 promoter. RORα

binding at six selected binding sites was validated with ChIP-PCR (Figure 4.10A) and

also with another RORα antibody recognizing a different epitope (Figure 4.10B).

Binding sites 1-3 are competing sites, and binding sites 4-6 are non-competing sites. All 6

bindings are indeed specific to RORα, as RORα binding at these sites was significantly

decreased in the RORα sg/sg mice carrying a nonsense mutation in the RORα gene

(Figure 4.10C, D).

We then compared REV-ERBα binding at the competing sites, non-competing sites and

in-phase sites, and found that REV-ERBα binding oscillated with similar amplitudes in

all three groups (Figure 4.9C). To understand the mechanisms that account for different

RORα binding rhythms, we performed *de novo* motif analysis on the competing sites

using the non-competiting sites as control. The top two motifs found were a DR2 nuclear

receptor motif, which is a direct repeat of the nuclear receptor half sites separated by two

nucleotides and a canonical RORα/REV-ERBα motif, which is a nuclear receptor half

site preceded by A/T-rich sequence (Figure 4.11A). Both motifs occurred at a

significantly higher frequency in the competing sites compared with the non-competing

or in-phase sites (Figure 4.11B). They were also significantly enriched near the center of

the RORα peaks, and the enrichment was much higher in the competing sites (Figure

4.11C, D). These findings suggest that competition between RORα and REV-ERBs is

more likely to occur at genomic regions with desired DNA sequences and high affinity

for RORα and REV-ERBs, and agree with our hypothesis that RORα and REV-ERBs

compete for the same binding motif *in vivo*. Moreover, the DR2 motif was enriched at the

center of the competing sites, but barely at the center of non-competing or in-phase sites (Figure 4.11D). This may be explained by the *in vitro* finding that REV-ERBα binds the DR2 motif as a homodimer, 5 to 10 times more stable than REV-ERBα monomer binding to the RORα motif (Harding and Lazar, 1993). The strong binding at the DR2 motif by REV-ERBs can better prevent RORα binding.

4.3d Overexpression of RORα Fails to Overcome REV-ERB Competition

To further test the hypothesis that REV-ERBs suppress RORα binding through direct competition, we overexpressed RORα4, the dominant RORα isoform in liver, using an adeno-associated virus encoding the RORα4 cDNA (AAV-RORα4). Two weeks after virus injection, we observed a 15 fold increase in the RORα4 transcript (Figure 4.12A). RORα4 overexpression increased RORα binding at ZT22 at all four binding sites when Rev-erbα is absent, including two new sites identified by ChIP-seq (Figure 4.12B), but failed to overcome REV-ERBα suppression at ZT10 at Bmal1, Npas2 and the RORα binding site 3, a competing site. In consistent with unchanged RORα binding, RORα4 overexpression failed to induce Bmal1 or Npas2 expression at ZT10 (Figure 4.12A). Our results suggest that REV-ERBs might have a higher affinity for the competing sites, and dominate in the competition with RORα. At the RORα binding site 4, a non-competing site, the increased RORα level doubled the RORα binding at both ZT10 and ZT22 (Figure 4.12B), supporting our model that REV-ERBs do not compete with RORα at these sites.

4.3e RORα Binding Correlates with Histone Acetylation

Nuclear receptors regulate transcription through recruiting coactivator and corepressor complexes. Coactivator complexes usually contain histone acetyltransferases (HATs), such as p300 and CBP. RORα has been shown to recruit p300 in muscle and coactivator SRC-2 in liver (Chopra et al., 2008; Lau et al., 1999). We discovered that p300 recruitment to the RORα binding sites correlates with the rhythmic RORα binding (Figure 4.13A). We observed rhythmic p300 recruitment only at the competing sites, but not at the non-competing sites. Moreover, the rhythmic p300 recruitment at the competing sites may allow a more robust oscillation in histone acetylation, in comparison with the non-competing sites (Figure 4.13B). Our findings suggest that in addition to HDAC3 recruitment, REV-ERBs may also control histone acetylation by repressing RORα-mediated recruitment of p300.

4.4 Discussion and Future Directions

In consistent with our finding that REV-ERBβ protects circadian rhythms in the absence of REV-ERBα, Evans and colleagues showed that Rev-erbα and Rev-erbβ double knockout mice become arrhythmic in constant darkness. So far every single component of the core clock machinery has been deleted genetically in mouse. Except for Bmal1 deletion (Bunger et al., 2000), none of the single mutation rendered mice arrhythmic in constant darkness, though most of them did affect the rhythm to some extent (Cermakian et al., 2001; DeBruyne et al., 2006; Dudley et al., 2003; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 1999, 2001). Even the Bmal1 deletion can be

compensated by ectopic expression of Bmal2 (Shi et al., 2010). In contrast, combinational deletion of the redundant components completely abolished the circadian rhythms, such as the double deletion of Clock and Npas2, Cry1 and Cry 2, Per1 and Per2, and Rev-erbα and Rev-erbβ (Bae et al., 2001; Cho et al., 2012; DeBruyne et al., 2007; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 2001). Together, these studies demonstrate the unusual redundancy of the core clock components. The circadian rhythm is so critical for survival that every component in the clock machinery needs a backup.

Although the paralogs in the circadian clock are redundant in maintaining circadian rhythms, they may be regulated differently and have different functions in circadian outputs. Our study showed that REV-ERBα and REV-ERBβ have almost identical genomic binding profiles and rhythms and a similar role in preventing hepatosteatosis. But they may behave differently in response to environmental signals. It may be interesting to see if the Rev-erbα-deficient mice and the Rev-erbβ-deficient show different response to light exposure or fasting/feeding. Also we should look into other tissues to see if the two REV-ERBs have different roles in regulating other physiological activities.

We identified three different types of RORα binding sites based on their binding rhythms: the competing sites, the non-competing sites and the in-phase sites. For the competing sites, as represented by the Bmal1 promoter and the Npas2 promoter, we showed that REV-ERBs repress RORα binding at ZT10 and drive a rhythmic RORα binding in

mouse liver. These binding sites are more likely to contain the RORα motif and the REV-ERBα DR2 motif, suggesting that competition happens in the genomic region with desired DNA sequences that allow specific and strong binding of RORα and REV-ERBα. We also discovered that rhythmic RORα binding correlates with p300 recruitment and contributes to the robust oscillation of histone acetylation at the competing sites. Our findings all agreed with our hypothesis that REV-ERBs drive circadian histone acetylation and gene expression through competing with RORα. We still need to address whether p300 recruitment is dependent on RORα.

So far, we have not addressed the biological significance of RORα. Previous studies in our lab showed that loss of HDAC3 does not abolish circadian rhythms as tested in MEFs and in liver (data not shown). Since the active repression model cannot account for all the activities of REV-ERBs, we hypothesized that passive repression through RORα also plays an important role in regulating circadian rhythms and metabolic activities. In consistent with our hypothesis, we found competing sites near clock genes including Bmal1, Npas2 and Cry1, metabolic genes including G6pase, Elovl5, Gpam and Hmgcs2, and metabolic regulators including PGC-1β, E4bp4 and Insig2 (data not shown). Disregulation of circadian and metabolic gene expression has been shown in the livers of the RORα sg/sg mice (Kang et al., 2007; Takeda et al., 2012). However, these mice suffer from severe impairment of orientation and motor capabilities and have significant lower weight compared to the WT mice, and therefore are not a good model to study the function of RORα in circadian rhythms and liver metabolism. Instead, we will use a

RORα conditional knockout model and delete RORα specifically in liver by injection of the AAV-Cre virus.

RORγ1 is also expressed in liver, and its transcript level is comparable to that of RORα (data not shown). Unlike RORα, RORγ1 shows circadian expression at the transcript level, which has not been validated at the protein level (Mongrain et al., 2008). Given that RORγ1 is expressed anti-phase to REV-ERBs, competition between REV-ERBs and RORγ1 may not exist or has little effect on RORγ1 activity. However, RORγ1 may compensate for the loss of RORα in liver. Therefore we will cross the RORα conditional knockout mice with the RORγ conditional knockout mice to generate the RORα/RORγ double conditional knockout mice to test the function of RORs in circadian rhythms and metabolism. Moreover, we may also discover non-overlapping functions of RORα and RORγ.

So far our studies have been focusing on the competing sites. However, it is also important to understand why REV-ERBs do not compete with RORα at the non-competing sites. ChIP-PCR showed relatively weak but real binding of RORα at the non-competing sites (Figure 4.10), and binding was doubled upon RORα overexpression at both ZT10 and ZT22. There may be two explanations for our observations. First, REV-ERBs and RORα may bind to two different motifs adjacent to each other. We can test this hypothesis by measuring the peak distance between the overlapping RORα and REV-ERB binding sites in the non-competing sites and comparing it to that in the competing sites. We can also try to identify multiple nuclear receptor motifs within the

97

non-competing sites. The second explanation is that the non-competing sites contain less than perfect motifs and have an open chromatin structure so that nuclear receptors like RORα and REV-ERBs can bind to them transiently and do not compete with each other. But such transient binding may not be important for gene regulation.

It is also intriguing that RORα is recruited in-phase with REV-ERBs at many sites and that these sites are significantly enriched for GR motif (Figure 4.11B, E). Obviously we need to validate the RORα binding at these sites. But it is interesting to see that GR binding is indeed enriched at these sites compared with the competing and non-competing sites (Figure 4.14). GR is stably expressed in liver, but its genomic binding and transcriptional activity is regulated by ligands, such as corticosterone. The plasma corticosterone level fluctuates with a peak between ZT10 and ZT14 and a trough between ZT20 and ZT24 (Oster et al., 2006). This explains our finding that GR binds to the mouse liver genome with a rhythm that is high at ZT10 and low at ZT22 (data not shown). We thus hypothesized that GR contributes to the in-phase binding of RORα. It is known that daily injection of glucocorticoid can drive the oscillation of 60% of the circadian transcriptome in liver, and it is possible that RORα is involved in this process. Once we validate the in-phase binding of RORα, we can test the GR hypothesis by activating GR binding at ZT22 with dexamethasone injection.

We reported that competition exists between REV-ERBs and RORα, but it may also exist between REV-ERBs and another nuclear receptor, such as PPARα, or between two other nuclear receptors. Mandrup and colleagues recently reported that PPARα binds to over

70% of the LXR sites in mouse liver, and binding of PPARα and LXR are mutually exclusive (Boergesen et al., 2012). Therefore, it may be interesting to compare the RORα cistrome and the REV-ERB cistrome we have with the public available cistromes of other nuclear receptors.

Recent advances in the next generation sequencing technology allowed scientists to accurately map the genome-wide binding of a large number of transcription factors in a variety of cell lines and tissues from different organisms. Comparing the transcription factor maps led to surprising findings that transcription factor binding is highly tissue-specific, and that in the same tissue or cell line, many transcription factors share a large number of genomic regions. For instance, Mandrup and colleagues recently reported that CEBPβ, STAT5a and GR co-occupy 'hotspots' characterized by an open chromatin structure and specific epigenetic modifications in early adipogenesis (Siersbæk et al., 2011). Pu and colleagues showed that several cardiac transcription factors, including GATA4, NKX2-5, MEF2A, TBX5 and SRF, co-occupy many genomic regions (He et al., 2011). Ng and colleagues discovered hundreds of multiple-transcription factor binding sites in embryonic stem cells (Chen et al., 2008). The modENCODE consortium reported high occupancy target (HOT) regions that are occupied by at least 15 transcription factors in *C. elegans* or by at least 8 transcription factors in Drosophila (Gerstein et al., 2010; Nègre et al., 2011). The molecular mechanisms and the biological significance of co-occupancy by transcription factors remain to be explored.
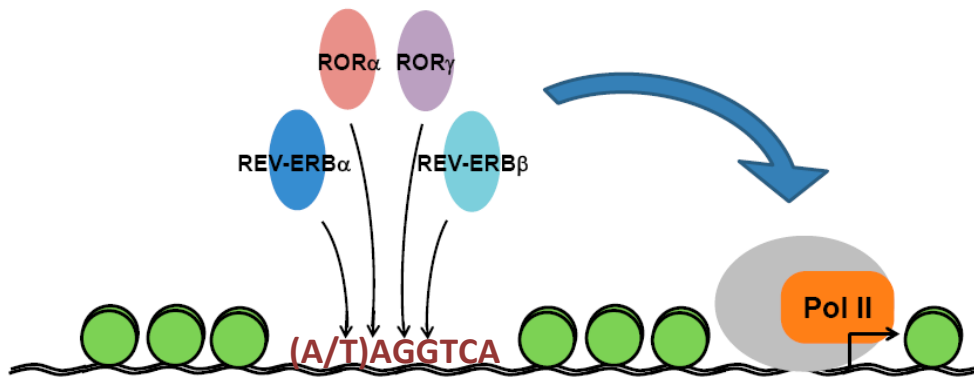
Figure 4.1 REV-ERBs and RORs Share Many Genomic Binding Sites. REV-ERBs and RORs recognize the same binding motif and share many gene targets including Bmal1 and Npas2.
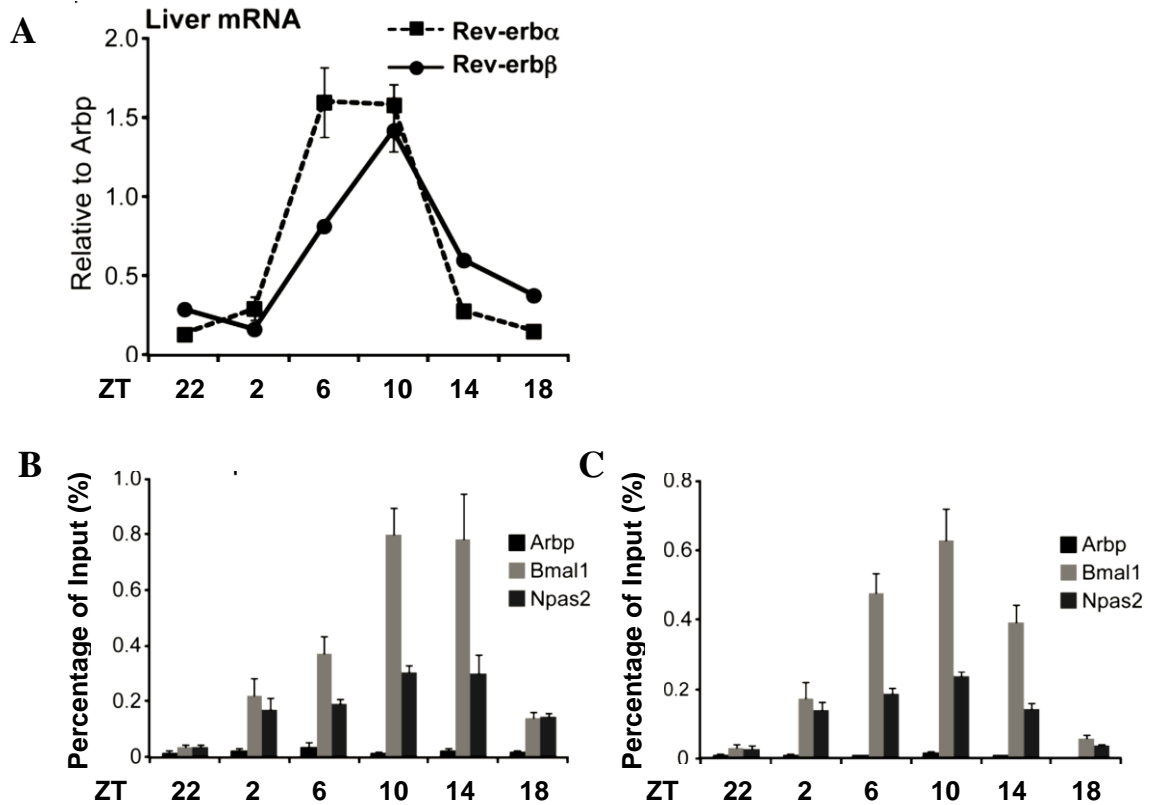
Figure 4.2 Circadian Expression and Genomic Binding of REV-ERBβ in Mouse Liver.

A. Rev-erbα and Rev-erbβ mRNA levels in the livers of 12 week old C57Bl/6 mice housed in a 24h cycle. Values are mean ± s.e.m (n=5).

B. REV-ERBβ recruitment at the Bmal1 promoter and the Npas2 promoter over a 24h cycle as interrogated by ChIP-PCR. Immunoprecipitated DNA was normalized to input. Values are mean ± s.e.m (n=3-4).

C. REV-ERBα recruitment at the Bmal1 promoter and Npas2 promoter over a 24h cycle as interrogated by ChIP-PCR. Values are mean ± s.e.m (n=3-4).
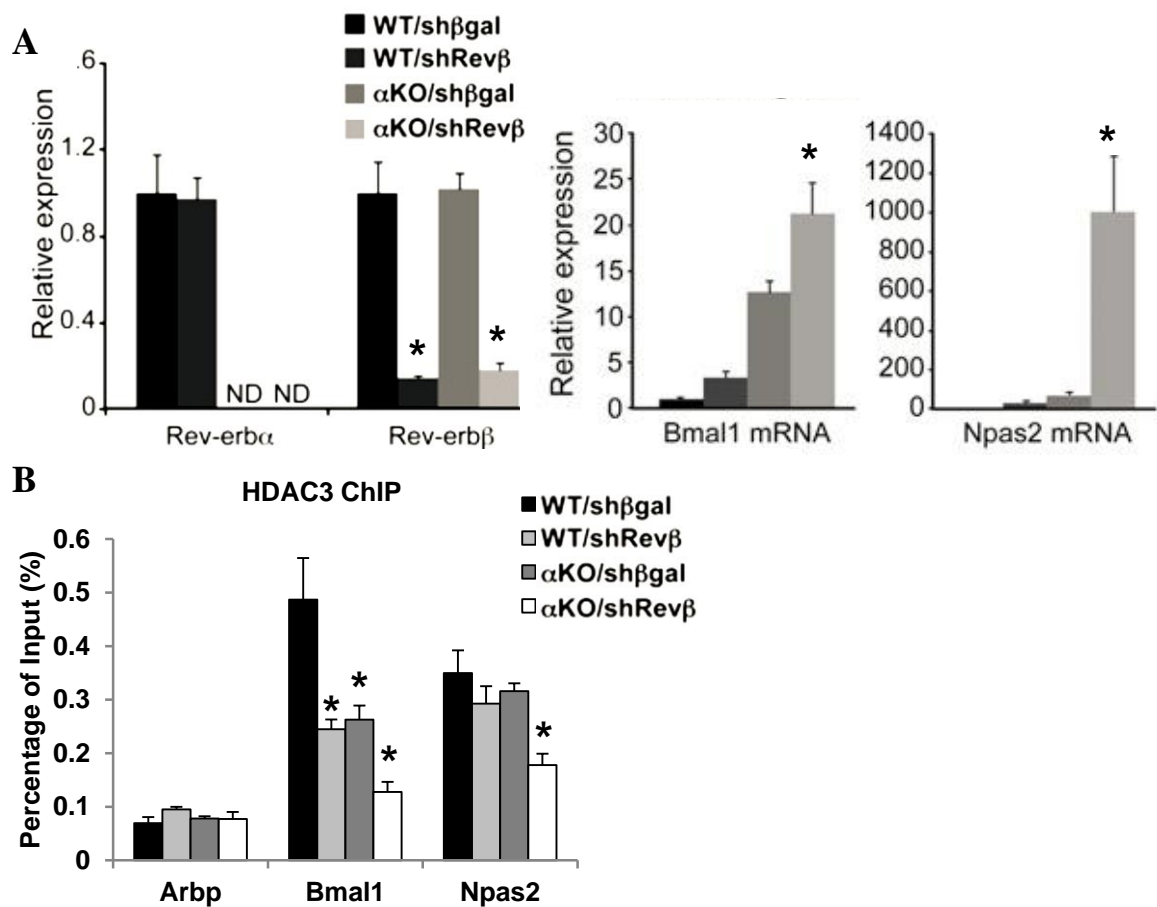
Figure 4.3 REV-ERBβ Compensates for Loss of REV-ERBα and Represses Clock Genes.

A. Gene expression in livers from 12 week old male WT or Rev-erbα knockout (αKO) mice one week after injection of adeno-shRev-erbβ (shRevβ) or adeno-shβGal (shβGal) as control. Values are mean ± s.e.m (n=4-6). *p<0.05 by student t-test.

B. HDAC3 recruitment in livers as in A as interrogated by ChIP-PCR. Values are mean ± s.e.m (n=3). *p<0.05 by student t-test.
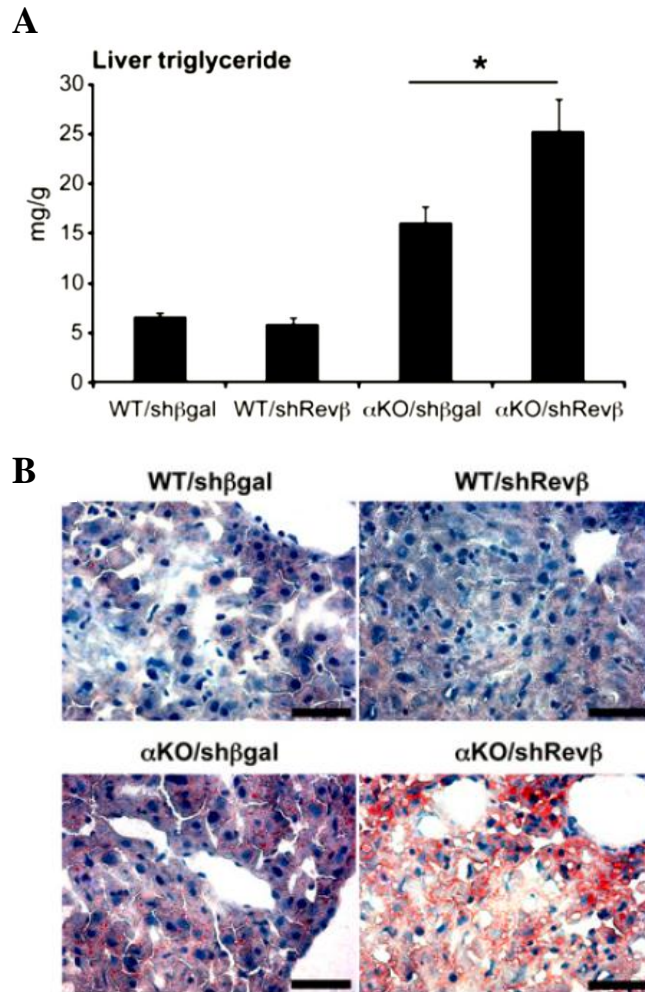
Figure 4.4 REV-ERBβ Prevents Liver Steatosis from Loss of REV-ERBα.

A. Hepatic TG levels in livers from 12 week old male WT or Rev-erbα knockout (αKO) mice one week after injection of adeno-shRev-erbβ (shRevβ) or adeno-shβGal (shβGal) as control. Values are mean ± s.e.m (n=5-7). *p<0.05 by student t-test.

B. Oil Red O staining of liver from 12 week old male WT or Rev-erbα knockout (αKO) mice one week after injection of adeno-shRev-erbβ (shRevβ) or adeno-shβGal (shβGal) as control.
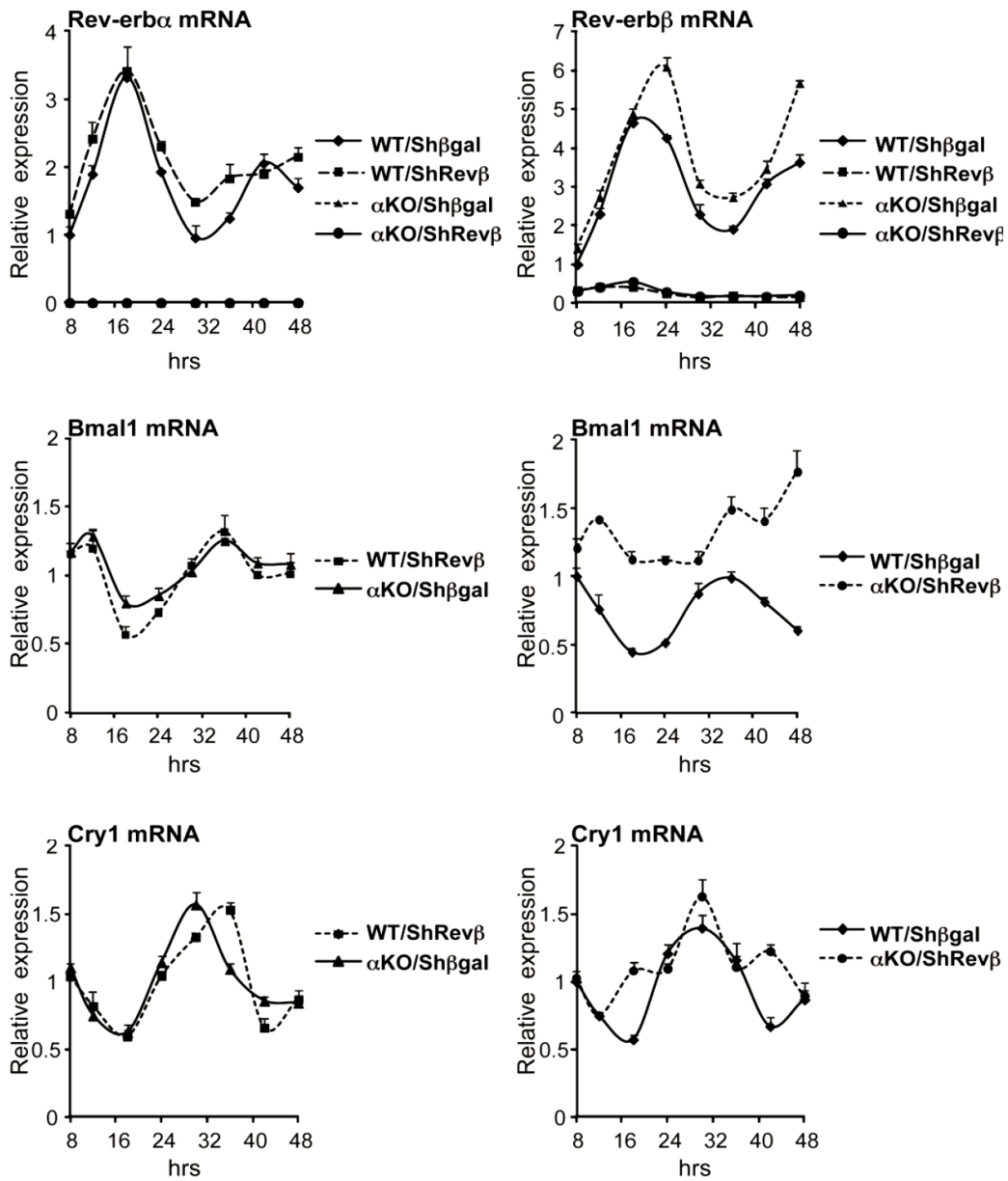
Figure 4.5 REV-ERBβ Protects the Circadian Clock from Loss of REV-ERBα.

Gene expression in synchronized WT or Rev-erbα knockout (αKO) MEFs infected with adeno-shRev-erbβ (shRevβ) or adeno-shβGal (shβGal) as control. Values are mean ± s.e.m (n=3). All values have been normalized to Arbp expression and the 8 hr time point in the WT control.
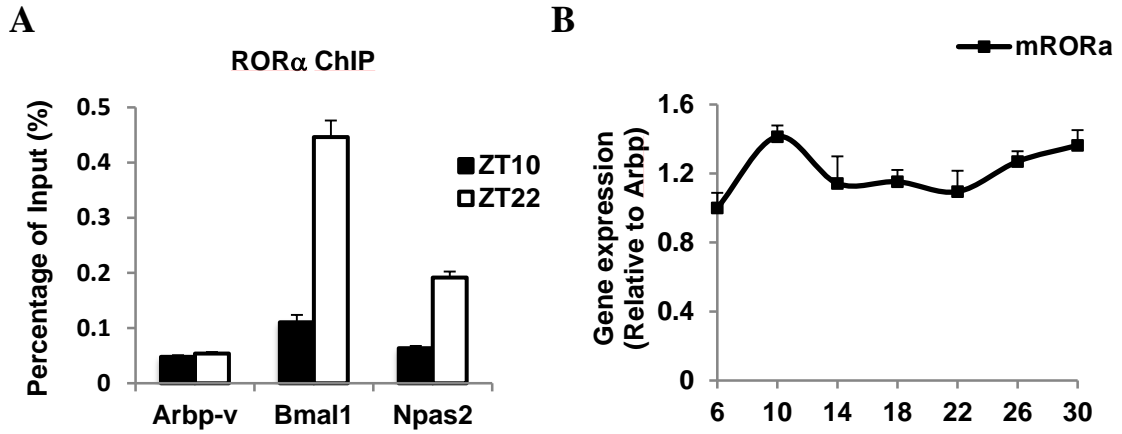
Figure 4.6 Diurnal Binding of RORα.

A. Diurnal recruitment of RORα to the Bmal1 promoter and the Npas2 promoter was shown by ChIP-PCR. The region close to the TSS of the Arbp gene served as a negative control. Values are mean ± s.e.m. (n=4-5).

B. RORα mRNA levels in the livers of 12 week old C57Bl/6 mice housed in a 24h cycle. Values are mean ± s.e.m (n=5).
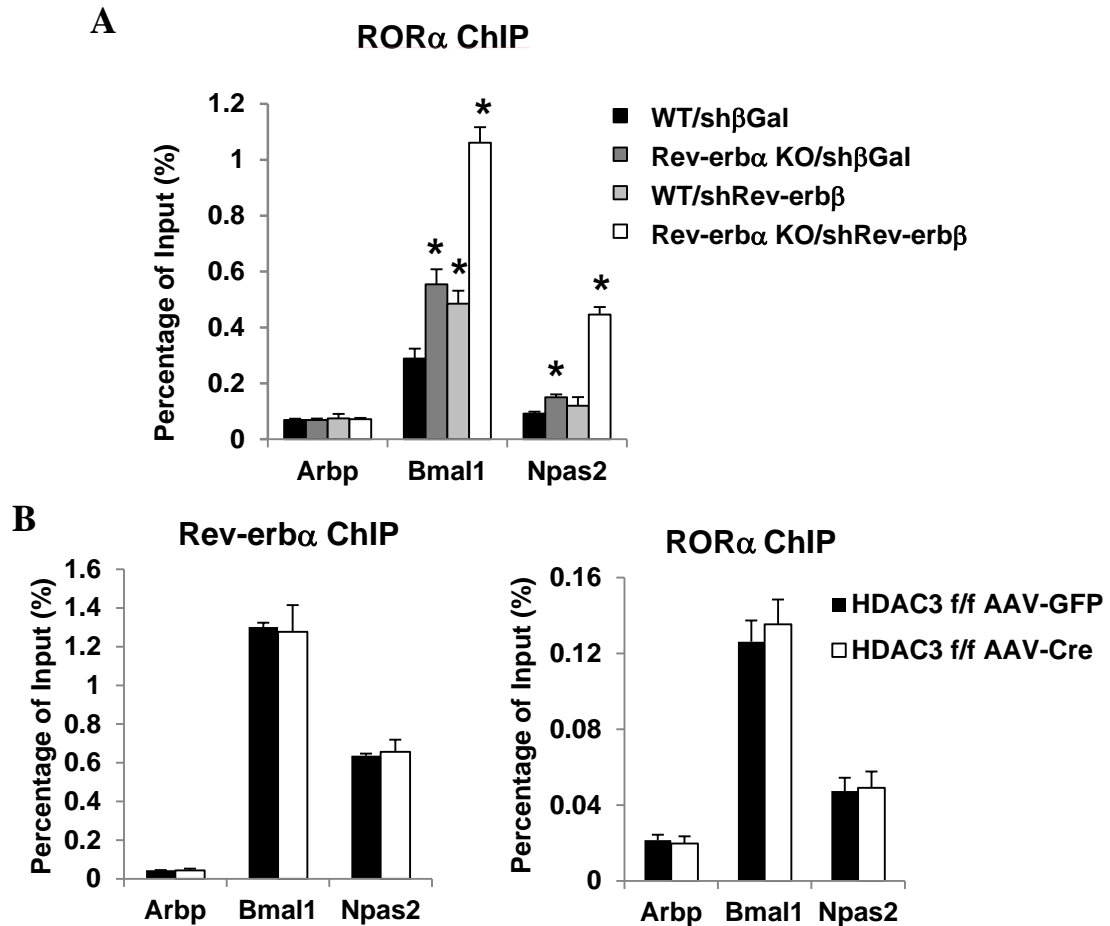
Figure 4.7 REV-ERBs Repress RORα Binding.

A. RORα recruitment to the Bmal1 promoter and the Npas2 promoter was upregulated in the absence of REV-ERBα and/or REV-ERBβ. RORα recruitment in livers from 12 week old male WT or Rev-erbα knockout (αKO) mice one week after injection of adeno-shRev-erbβ (shRevβ) or adeno-shβGal (shβGal) was interrogated by ChIP-PCR. Values are mean ± s.e.m (n=4-5). *p<0.05 by student t-test.

B. RORα recruitment to the Bmal1 promoter and the Npas2 promoter was unaffected in the absence of HDAC3. REV-ERBα and RORα recruitment in livers from the HDAC3$^{fl/fl}$ mice 1 week after injection of the AAV-Cre virus was interrogated by ChIP-PCR. AAV-GFP virus was used as a negative control. Values are mean ± s.e.m (n=4).
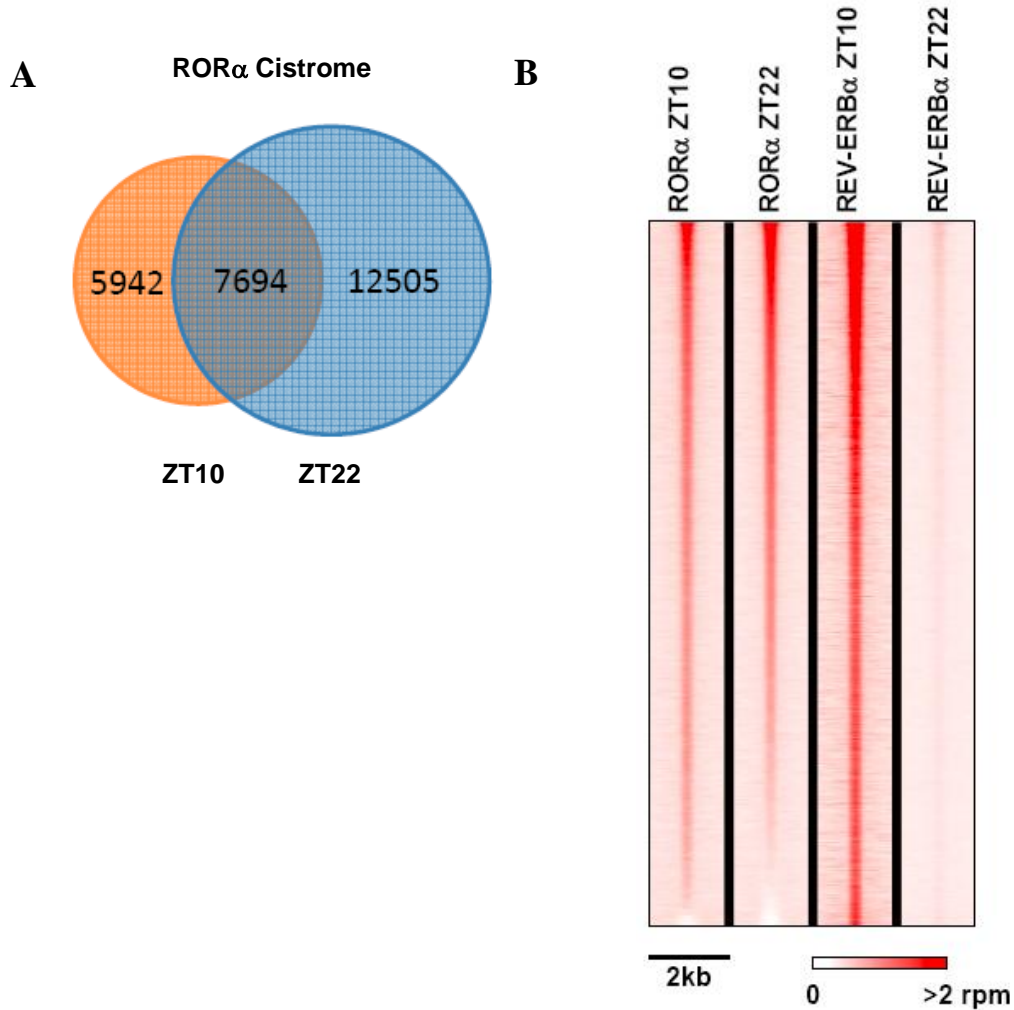
Figure 4.8 RORα Cistrome in Liver at ZT10 and ZT22.

A. Comparison of HDAC3 binding sites at ZT10 (Blue) and ZT22 (Red). Peak calling was performed with HOMER. A pair of ZT10 and ZT22 binding sites (approximately 200bp wide) was considered overlapping when they have at least 1bp in common.

B. Heatmap of RORα and REV-ERBα binding signal at ZT10 and ZT22 from -1kb to +1kb surrounding the center of all the RORα and REV-ERBα binding sites. Each line represents a single binding site and the color scale indicates the binding signal (read density in ChIP-seq data).

A RORα

Log Peak Density at ZT22

Log Peak Density at ZT10

B

0-30 REV-ERBα ZT10

0-30 REV-ERBα ZT22

0-30 RORα ZT10

0-30 RORα ZT22

Arntl

C

| Competing Sites | Non-competing | In-phase Sites |

RORα read density

REV-ERBα read density
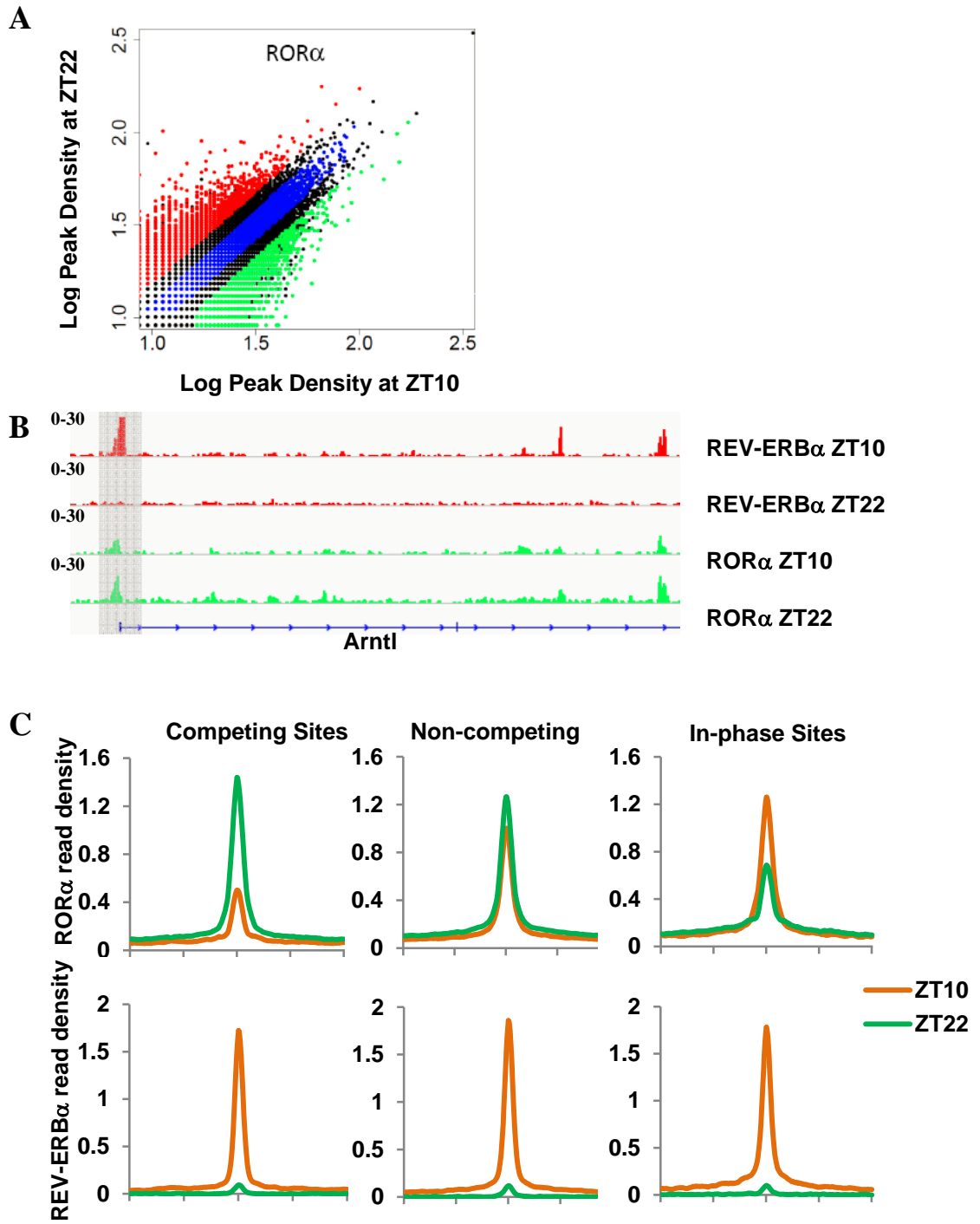
ZT10
ZT22

Figure 4.9 Identify Competing, Non-competing and In-phase Binding Sites.

A. Scatter plot of RORα binding signals at ZT10 (x-axis) and ZT22 (y-axis) in log scale. Competing (Red), no-competing (Blue) and in-phase (Green) sites were identified based on the fold changes of signals from ZT10 to ZT22.

B. The Bmal1 promoter was identified as a competing site. Peak height correlates with the REV-ERBα (Red) or RORα (Green) ChIP-seq signal at ZT10 or ZT22.

C. Average RORα and REV-ERBα binding signals surrounding the center of the competing, non-competing and in-phase sites at ZT10 and ZT22. The signals were represented by the read density in ChIP-seq data.
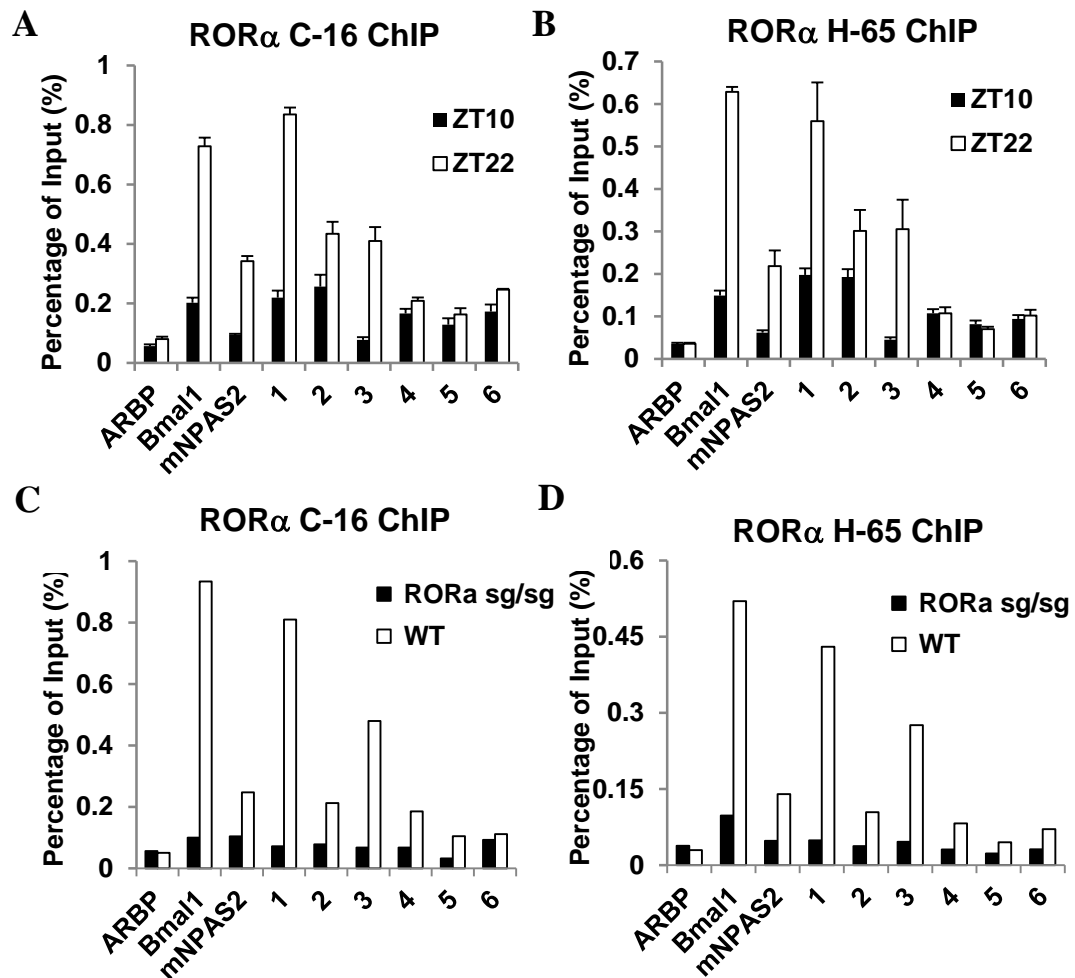
Figure 4.10 Validation of RORα Binding Sites.

A. B. RORα recruitment at ZT10 and ZT22 as shown by ChIP-PCR with two different RORα antibodies C-16 (**A**) and H-65 (**B**). Six RORα binding sites were interrogated, including 3 competing sites (1-3) and 3 non-competing sites (4-6). The Bmal1 gene promoter and the Npas2 promoter were used as positive controls, and the region close to the Arbp TSS as a negative control. Values are mean ± s.e.m. (n=4).

C. D. RORα recruitment at ZT22 in livers from WT mice or the RORα sg/sg mice as shown by ChIP-PCR with two different RORα antibodies C-16 (**C**) and H-65 (**D**).
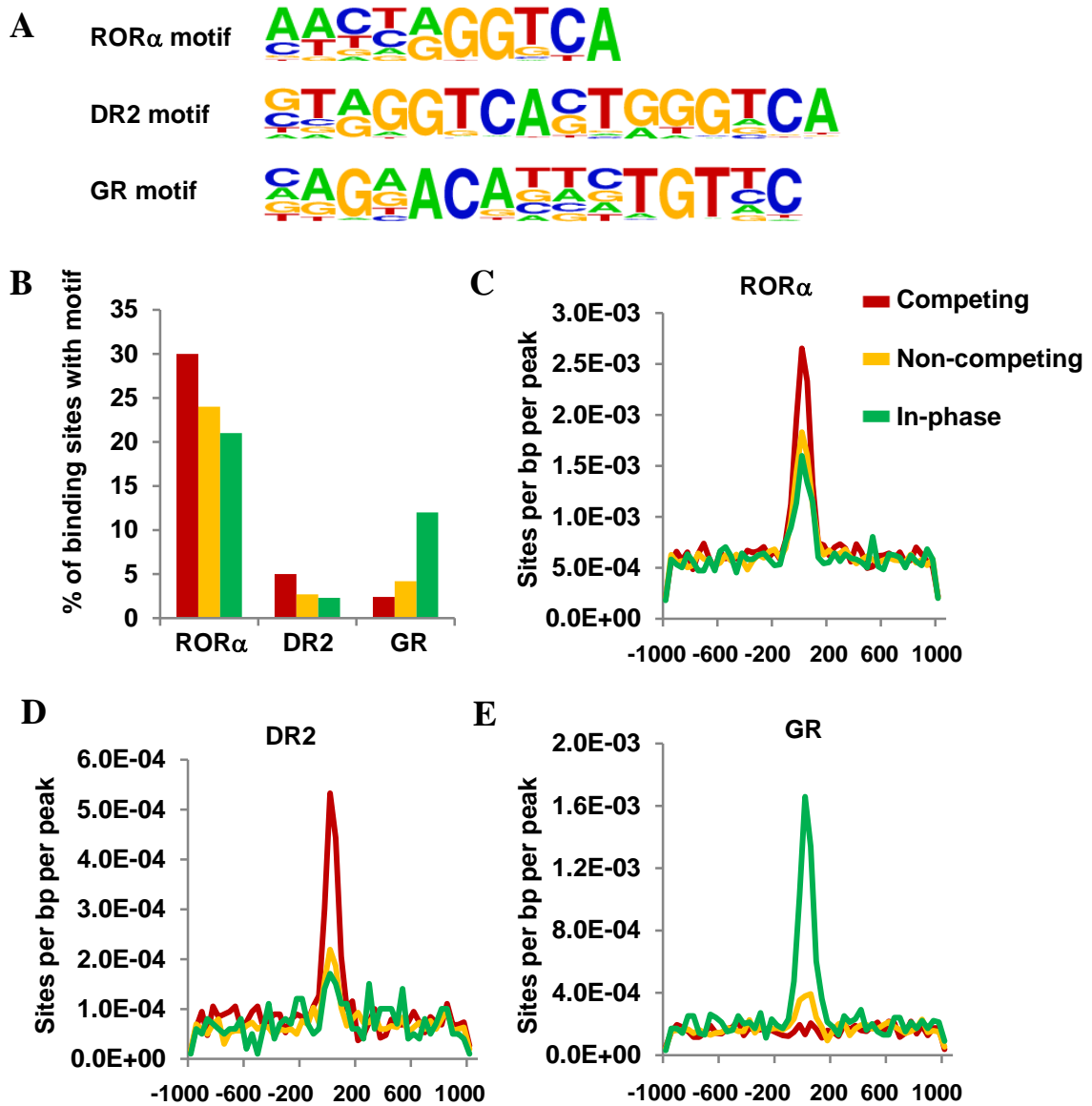
Figure 4.11 Motif Analysis of the RORα Binding Sites.

A. Three motifs identified in motif analysis of the competing, non-competing and in-phase binding sites.

B. Percentage of bindings sites with either RORα, DR2 or GR motif as shown in **A** among the competing (Red), non-competing (Yellow) and in-phase (Green) binding sites.

C. D. E. Density of the RORα (**C**), DR2 (**D**) and GR (**E**) motif surrounding the center of the competing (Red), non-competing (Yellow) and in-phase (Green) binding sites.
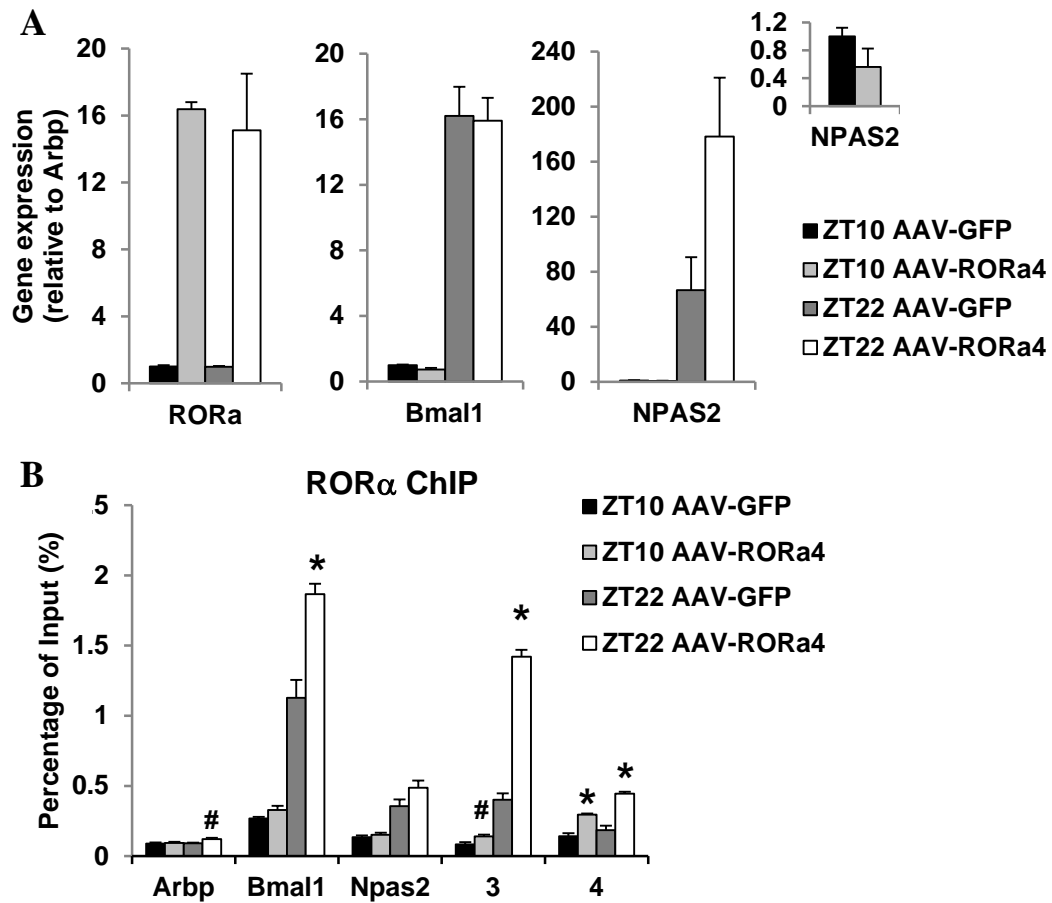
Figure 4.12 Overexpression of RORα Fails to Overcome REV-ERB Competition.

A. RORα, Bmal1 and Npas2 expression in livers harvested at ZT10 and ZT22 from 12 week old C57Bl/6 mice 2 weeks after injection of AAV-RORα4 or AAV-GFP (control). Values are mean ± s.e.m (n=3).

B. RORα recruitment in livers harvested at ZT10 and ZT22 from 12 week old C57Bl/6 mice 2 weeks after injection of AAV-RORα4 or AAV-GFP (control). Values are mean ± s.e.m (n=3). *p<0.05; #p~0.05.
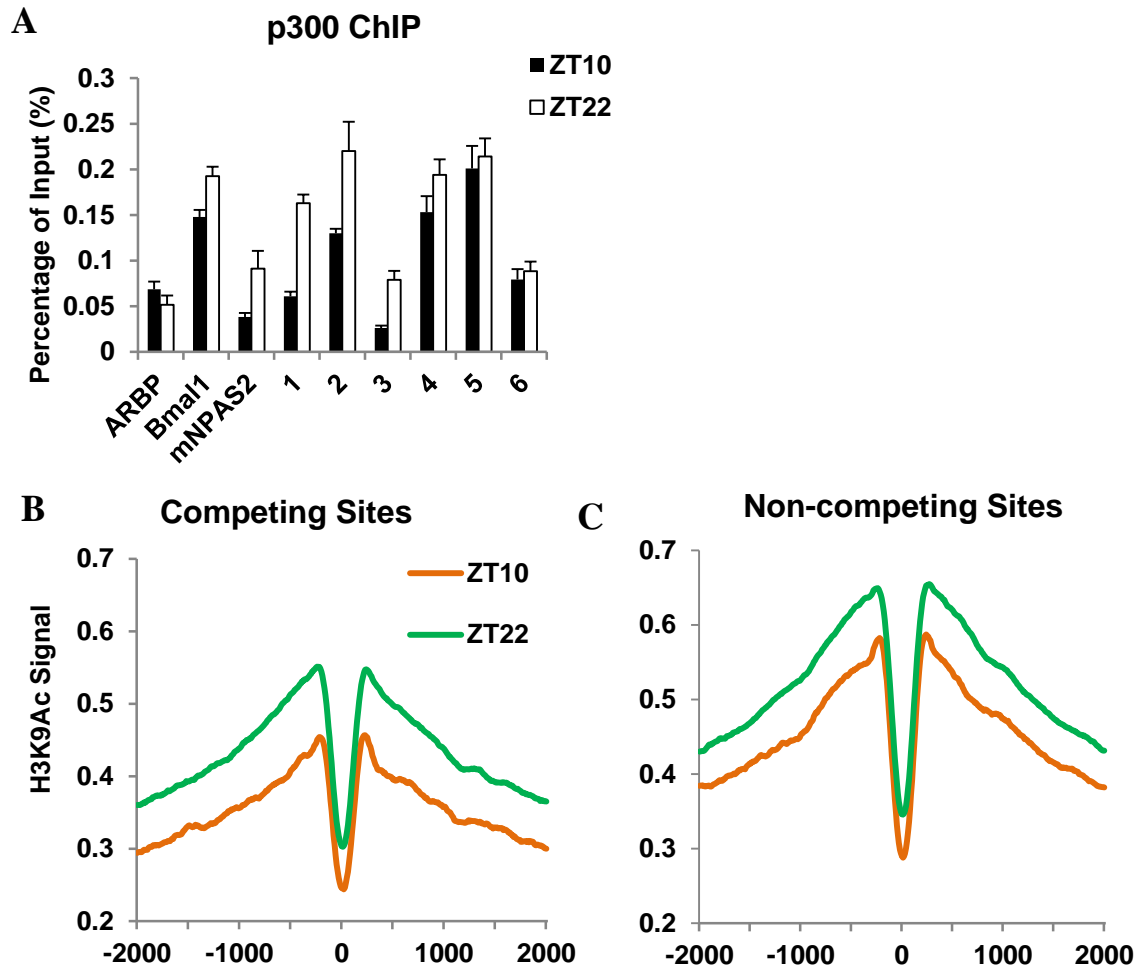
Figure 4.13 RORα Binding Is Associated with p300 Recruitment and Histone Acetylation.

A. p300 recruitment at ZT10 and ZT22 as shown by ChIP-PCR at six RORα binding sites, including 3 competing sites (1-3) and 3 non-competing sites (4-6). The Bmal1 gene promoter and the Npas2 promoter were used as positive controls, and the region close to the Arbp TSS as a negative control. Values are mean ± s.e.m. (n=4).

B. Average H3K9Ac signal from -2kb to +2kb surrounding the center of all the competing (Left) and non-competing sites (Right). The Y axis represents the H3K9Ac signal (read density).
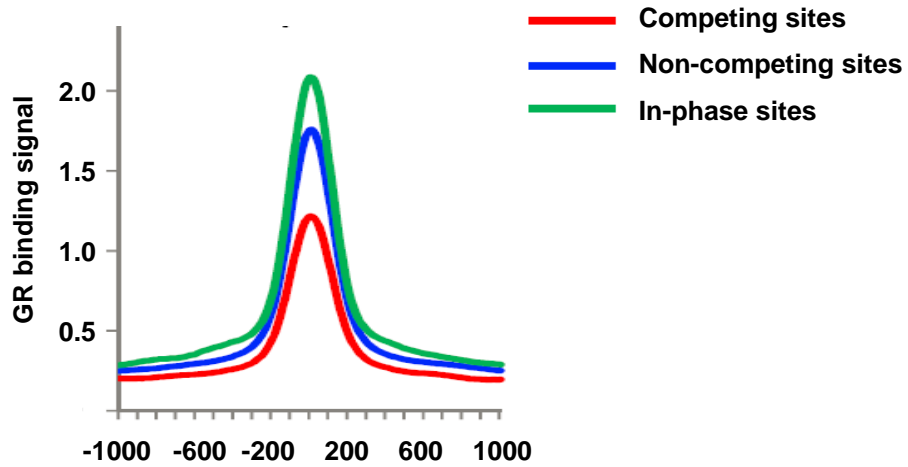
Figure 4.14 In-phase RORα Binding sites Are Enriched for GR binding. Average GR binding signal at ZT10 from -1kb to +1kb surrounding the center of all the competing (Red), non-competing sites (blue) and in-phase (Green) sites. The Y axis represents the GR signal (read density).

Chapter 5

Future Directions

5.1 Summary

My study suggests that REV-ERBα and REV-ERBβ direct a circadian rhythm in histone acetylation and gene expression that is required for hepatic homeostasis in liver, by recruiting histone deacetylase HDAC3 or suppressing RORα and histone acetylase recruitment. In Chapter 3, I showed that histone deacetylase 3 (HDAC3) recruitment to the genome displays a circadian rhythm in mouse liver. Histone acetylation is inversely correlated to HDAC3 binding, and this rhythm is lost in the absence of HDAC3. Although protein amounts of HDAC3 are constant in liver, its genomic recruitment corresponds to the expression pattern of the circadian nuclear receptor REV-ERBα. I demonstrated that REV-ERBα is required for HDAC3 recruitment at many HDAC3 binding sites. I found that REV-ERBα colocalizes with HDAC3 near genes regulating lipid metabolism, and deletion of HDAC3 or REV-ERBα in mouse liver causes hepatic steatosis. Therefore, I propose that the genomic recruitment of HDAC3 by REV-ERBα directs a circadian rhythm of histone acetylation and gene expression required for normal hepatic lipid homeostasis.

In Chapter 4, I examined the role of REV-ERBβ and RORα, two nuclear receptors that are closely related to REV-ERBα. Rev-erbα deletion has modest effects on both circadian rhythms and lipid metabolism. Here I reported that REV-ERBβ displays circadian recruitment to the Bmal1 and Npas2 genes, similar to that of REV-ERBα. Depletion of REV-ERBβ in Rev-erbα null liver further decreases HDAC3 recruitment, derepresses Bmal1 and Npas2 expression, and exacerbates hepatic steatosis. I also showed that loss of both REV-ERBs abolishes the circadian rhythm in mouse embryonic

fibroblasts. Our findings established the two REV-ERBs as major regulators of both clock function and hepatic metabolism.

In the second half of Chapter 4, I reported that RORα binds the Bmal1 and Npas2 genes with a diurnal rhythm anti-phase to that of REV-ERBs. I showed that REV-ERBs repress RORα binding at ZT10 and thus drives the diurnal recruitment of RORα, which is expressed at constant levels. Repression by REV-ERBs is independent of HDAC3, suggesting that REV-ERBs represses RORα binding through direct competition. I then identified thousands of competing sites similar to those in Bmal1 and Npas2 genes by RORα ChIP-seq at ZT10 and ZT22, many of which are in close proximity of clock and metabolic genes. RORα function in circadian rhythms and hepatic metabolism will be tested in mouse models of liver-specific deletion of RORα and its paralog RORγ. I also discovered thousands of RORα binding sites with no rhythmic RORα binding, and sites where RORα bind is in phase with REV-ERBs. How RORα recruitment is regulated at these non-competing or in-phase sites remains to be elucidated.

5.2 Future Directions

So far I have shown that REV-ERBs direct circadian recruitment of HDAC3 and RORα, drive rhythmic histone acetylation, and contribute to circadian lipid biosynthesis and lipid homeostasis in liver. Although evidence suggests that diurnal recruitment of HDAC3 is regulated by the liver clock, which is the core clock machinery in hepatocytes, I have not yet demonstrated that HDAC3 recruitment is truly 'circadian' and is sustained in the absence of food intake. In addition, I have focused on how the liver clock regulates

metabolism, but not yet investigated how the metabolic state and metabolites regulate the clock through REV-ERBs. In the next step, I will explore how REV-ERB activity is modulated in liver, particularly by metabolic signals. In Chapter 3, I suggested that REV-ERBs and HDAC3 may be involved in the development of fatty liver disease in human caused by circadian misalignment, such as rotating shift work and jet-lag. I would like to test this hypothesis, and explore how circadian misalignment is associated with increased risk of metabolic diseases in human. Last but not least, I would like to investigate the non-redundant functions of REV-ERBα and REV-ERBβ, and further explore HDAC3 function in circadian rhythms.

5.2a Diurnal Recruitment of REV-ERBs and HDAC3—Clock or Food?

As I discussed in Section 1.5, the oscillating liver transcriptome (the once called 'circadian' transcriptome) is the result of the oscillating liver clock and the fasting/feeding cycles. Usually the feeding behavior is aligned with the liver clock, and they work together to drive rhythmic gene expression and metabolic activities. In the absence of feeding or food intake, most of the 'circadian' liver transcripts can no longer oscillate. In Chapter 3, I showed that the diurnal recruitment of HDAC3 is sustained in constant darkness but was reversed when restricted feeding reset the liver clock (Figure 3.6). These findings suggest that HDAC3 recruitment is regulated by the liver clock, the core clock machinery in hepatocytes. However, in both cases, the liver clock was still aligned with fasting/feeding cycles, and they could both contribute to the diurnal recruitment of HDAC3. Is the cyclic REV-ERB and HDAC3 recruitment driven solely by

the circadian clock or is it also regulated by food intake? To answer this question, I proposed the following experiments.

To prove that the cyclic REV-ERB and HDAC3 recruitment is driven by the circadian clock, I will isolate and synchronize mouse primary hepatocytes, and ask if the diurnal REV-ERB and HDAC3 recruitment sustains in a cell-autonomous manner. But this *in vitro* model has one major disadvantage that the isolated hepatocytes cannot maintain the differentiated state and quickly lose their metabolic capacity. Therefore, we may not be able to study how REV-ERBs and HDAC3 regulate metabolic genes and metabolic activities in these cells.

The second experiment was to test whether the diurnal recruitment of REV-ERBs and HDAC3 is sustained in the absence of food intake *in vivo*. In this experiment, mice were fasted from ZT10, and a subset of them was refed 18 hours later at ZT4. We interrogated REV-ERBα and HDAC3 recruitment in mouse liver at ZT22 (12-hour fasting) and at the next ZT10 (24-hour fasting or 6-hour refeeding after 18-hour fasting) (Figure 5.1A). In the absence of food (fasting), I observed significant increase in HDAC3 recruitment at ZT22 in most of the sites we tested, including the Bmal1 promoter, but the diurnal rhythm of HDAC3 recruitment was partially sustained (Figure 5.1C). This result indicates that diurnal HDAC recruitment is driven at least partially by the liver clock. REV-ERBα recruitment was also increased significantly at many sites, suggesting that REV-ERBα may contribute to the increase in HDAC3 recruitment (Figure 5.1B). However, the increase in REV-ERBα recruitment was much smaller than that in HDAC3

recruitment, and this difference may be explained by enhanced HDAC3 recruitment by REV-ERB upon fasting or by transcription factors other than REV-ERBs. On the contrary, feeding seems to decrease REV-ERBα and HDAC3 recruitment. I observed a significant decrease in both HDAC3 and REV-ERBα binding at ZT10 in the livers of the refed mice (Figure 5.1B,C). Together, these findings show that rhythmic REV-ERB and HDAC3 recruitment is regulated by the liver clock and by fasting/feeding. So far, I have only interrogated a small set of HDAC3 binding sites by ChIP-PCR, and observed different responses to fasting/feeding among these sites. I plan to expand my study genome-wide using ChIP-seq. I will also try to understand how REV-ERBα and HDAC3 recruitment is regulated by fasting/feeding and test whether changes in HDAC3 recruitment is independent of REV-ERBs.

5.2b Modulation of REV-ERBα Activity

So far, I have established the essential role of REV-ERBs in circadian rhythms and demonstrated a pivotal role of REV-ERBs in regulation of liver metabolism by the circadian clock. The next step is to understand how REV-ERBα activity is modulated by metabolic signals and others. As I discussed in Section 1.4, the crosstalk between circadian clocks and metabolism is not unidirectional, and metabolic signals can regulate clock gene expression and the transcription activity of the clock proteins (Figure 1.5). The feedback regulation is critical for metabolic homeostasis and fine tuning of the clock function. The role of REV-ERBα in this process remains to be explored.

First, heme is a REV-ERBα ligand (Yin et al., 2007). *In vitro* study showed that REV-ERB structure and corepressor recruitment can be regulated by the intracellular redox state and gases such as O_2, NO and CO through heme (Pardee et al., 2009; Yin et al., 2007). This suggests that heme binding to REV-ERBα may have important regulatory functions on circadian rhythms and hepatic metabolism. Does loss of heme binding really inhibit HDAC3 recruitment by REV-ERBα *in vivo*, and affect the clock and metabolic gene expression and lipid homeostasis? To answer this question, I plan to inhibit heme biosynthesis by suppressing the rate-limiting enzyme ALAS1 in mouse liver, and examine HDAC3 genomic recruitment, clock and metabolic gene expression and liver triglyceride in response to decrease in heme levels. Secondly, REV-ERBα protein stability and abundance is regulated by phosphorylation and ubiquitination. Is the post-translational regulation important for circadian REV-ERBα expression in mouse liver? This question can be addressed by ectopic expression of wildtype REV-ERBα or mutant REV-ERBα with serine to alanine mutations at varies phosphorylation sites. If my hypothesis is correct, I should observe oscillation in ectopically expressed wildtype REV-ERBα, and should find little or no mutant REV-ERBα in liver. Previously I showed that REV-ERBα binding was significantly down-regulated in the refed mice (Figure 5.1B). Does REV-ERBα protein level decrease? Is it through post-translational modification? I can address this question by measuring REV-ERBα protein levels and GSK3β activation in these livers.

5.2c Circadian Disruption and Liver Steatosis

Circadian clocks in our bodies synchronize metabolic events with our daily activity cycles, and therefore ensure the energy supply and maintain the internal homeostasis. But this delicate system has been increasingly challenged in modern society. Circadian rhythm disorders have been observed in a large population due to rotating shift work, jet-lag, night eating, sleep disruption and deprivation, and many epidemiological studies have shown that circadian rhythm disorders lead to a higher risk of metabolic diseases, including obesity, diabetes and non-alcoholic fatty liver disease. In recognition of these concerns, the Lazar lab studies the crosstalk of circadian clocks and metabolism through REV-ERBs, HDAC3s and RORs, and hopes to understand the pathogenic processes of metabolic disorders caused by circadian rhythm disorders/circadian misalignment.

In Chapter 3, I proposed that misalignment of fasting/feeding and sleep/wake cycles with endogenous circadian cycles could disrupt rhythmic HDAC3 association with lipid metabolic genes and contribute to the fatty liver observed in rotating shift workers as well as people with genetic variants of clock genes (Brunt, 2010; Sookoian et al., 2007). However, we do not have any evidence that circadian misalignment or genetic mutation of clock genes affects the circadian binding of HDAC3 and causes liver steatosis. And I plan to test this hypothesis using mouse models mimicking human circadian rhythm disorders or genetic mutation of clock genes.

In collaboration with Dr. Loning Fu at Baylor College of Medicine, I first studied a chronic jet-lag mouse model. Jet lag is a physiological condition that results from rapid

long-distance transmeridian travel (typically by jet). After landing in a new time zone, the circadian clock of the passenger is misaligned with the destination time. Both jet lag and shift work cause circadian misalignment, affect the body clock, and result in similar symptoms such as fatigue and loss of appetite (Waterhouse, 1999). In our model, mice were treated with chronic back-and-forth jet-lag since 4 weeks of age. At the beginning of each week, the light/dark cycle was advanced by 8 hours and stayed for 3 days, and was switched back and stayed for 4 days (Figure 5.2A). Consistent with the epidemiological evidence that shift workers may have a higher risk of liver steatosis, we observed a significant increase in liver triglyderide in the jet-lag mice 3 weeks after the treatmenet (data not shown, provided by Dr. Loning Fu). In the livers from the jet-lag mice, HDAC3 still binds genome sites at both ZT10 and ZT22, but the diurnal binding rhythm was completely abolished (Figure 5.1C). HDAC3 binding in the jet-lag mice at both ZT10 and ZT22 was lower than that at ZT10 in control mice, but higher than that at ZT22 in control mice. The intermediate binding of HDAC3 in the jet-lag mice was consistent with the intermediate expression of both REV-ERBs (Figure 5.2B). Using the jet-lag mouse model, I showed that chronic circadian misalignment impaired the circadian clock, abolished diurnal HDAC3 recruitment and caused liver steatosis.

I also tested my hypothesis with Cry1/2 double knock mice (Cry DKO), in which circadian rhythms are completely abolished (Van der Horst et al., 1999). These mice also showed a significant increase in liver fat content (data not shown, provided by Dr. Loning Fu). REV-ERBα binding rhythm was abolished, as gene expression no long oscillated in these mice (Figure 5.3A, B). Since HDAC3 is recruited by REV-ERBs, it too lost the

binding rhythm (Figure 5.3C). Here I showed that genetic mutation of the clock genes abolished diurnal HDAC3 recruitment and caused liver steatosis.

My studies on the jet-lag mice and the Cry DKO mice demonstrate that circadian misalignment or genetic mutation of clock genes affects the circadian binding of HDAC3 and causes liver steatosis. However, I have not yet been able to prove the cause and effect relationship between loss of rhythmic HDAC3 and REV-ERB binding and fatty liver. To establish the cause-and-effect relationship, I propose the following studies.

First, I will test whether loss of the liver circadian rhythm causes liver steatosis. Both chronic jet-lag and Cry1/2 double knockout impair the central clock and the peripheral clocks, and alter both behaviors and physiology. To focus on the liver clock, I will create a mouse model in which only the liver clock is "jet-lagged". A preliminary idea is to provide food only for 4 hours a day, and at the beginning of each week advance the feeding time for 8 hours and switch back 3 days later. The back-and-forth shift in timely restricted feeding should impair the rhythmic expression of clock genes in major metabolic organs, particularly liver, yet have no minimum effects on the central clock. I will then test whether the feeding regimen abolishes HDAC3 recruitment rhythm, affects circadian and metabolic gene expression, and causes increase in liver triglyceride content.

The key experiment to establish the cause-and-effect relationship is to ectopically express REV-ERBα and/or REV-ERBβ in liver to a stable intermediate level observed in the jet-lag mice. To eliminate the effects of endogenous REV-ERBs, I will perform this

experiment in mice with genetic deletion of both Rev-erbα/β in liver. These mice will be housed under regular light/dark cycles and allowed free access to food. If liver triglyceride is increased in mice with a stable intermediate level of REV-ERB, we may conclude that circadian misalignment abolishes rhythmic HDAC3 and REV-ERB binding, and thus leads to fatty liver. And this finding will strongly support the role of HDAC3 and REV-ERBs in the development of non-alcoholic fatty liver disease in human with circadian rhythm disorders.

In summary, my studies using the chronic jet-lag mouse model and the Cry DKO model agree with the hypothesis that the impaired HDAC3 binding rhythm is involved in the pathogenesis of fatty liver caused by circadian disruption. These mouse models mimic circadian misalignment in human, and therefore may provide useful insights into the onset of human fatty liver disease.

5.2d Non-redundant Roles of REV-ERBα and REV-ERBβ

In Chapter 4, I showed that REV-ERBα and REV-ERBβ have almost identical roles in transcriptional regulation, and overlapping functions in circadian rhythms and hepatic lipid metabolism. As a result, I have always been asked if these two always share the same role. The answer may be 'No'. First, REV-ERBα and REV-ERBβ have slightly different expression rhythms. Although they mostly bind the same genomic regions and are likely to share the same gene targets in liver, they may repress these targets at slightly different times of the day. Secondly, REV-ERBα and REV-ERBβ may be expressed at different abundance in liver. Therefore, one may be more important than the other in the

liver clock and liver metabolism. Last, REV-ERBα and REV-ERBβ bind to the Rev-erbα promoter, but not the Rev-erbβ promoter, and this difference hints that REV-ERBα and REV-ERBβ are not equal in the core clock machinery. Indeed, genetic deletion of Rev-erbα has a more significant effect on circadian rhythms compared with genetic deletion of Rev-erbβ (Cho et al., 2012). To compare the roles of REV-ERBα and REV-ERBβ in the liver, I plan to use the conditional null models for both genes, and carefully examine gene expression changes in response to liver-specific deletion of REV-ERBα and/or REV-ERBβ. In Chapter 3 and 4, I tested the function of REV-ERBα and REV-ERBβ on circadian rhythms and hepatic metabolism using germline deletion of Rev-erbα or an adenovirus that encodes shRNA targeting Rev-erbβ. But I cannot accurately compare the roles of REV-ERBα and REV-ERBβ by using two different methods, not to mention that each method has major drawbacks. Germline deletion leads to complete loss of Rev-erbα throughout the body, and adenovirus encoding shRNA has off-target effects and induces immune response and liver toxicity. Aware of these drawbacks, I also hope to validate all my previous findings using the conditional null models for Rev-erbs.

Although REV-ERBα and REV-ERBβ may share the same genomic binding and gene targets, they could respond differently to cell signaling and have different physiological roles. For instance, REV-ERBα is subject to post-translational regulation by GSK3β and E3 ubiquitin ligase ARF-BP/PAM. GSK3β phosphorylates serine 55 and 59 in the N-terminal domain of REV-ERBα, and protects REV-ERBα from ubiquitination and degradation. The GSK3β phosphorylation motif is not conserved in REV-ERBβ. This

raises two possibilities. First, loss of REV-ERBα affects a subset of metabolic genes, and REV-ERBβ protects the rest metabolic pathways and the circadian clock. This could be addressed by carefully comparing the Rev-erb conditional null models discussed earlier. Second, REV-ERBα, but not REV-ERBβ, receives environmental signals and adjusts the liver clock. This is supported by a previous finding in the lab that serum shock induces REV-ERBα degradation through ARF-BP/PAM.

In summary, I would like to address the non-redundant functions of REV-ERBα and REV-ERBβ in liver by comparing gene expression in response to loss of either REV-ERB, and by studying how REV-ERBs are differentially regulated in response to systematic signals, such as insulin, glucocorticoid and serum shock. I also plan to test if the redundancy of REV-ERBα and REV-ERBβ observed in liver also exists in other major metabolic organs, such as adipose tissues and muscle.

5.2e HDAC3 in Circadian Rhythms and Others

HDAC inhibitors are widely used in clinical trials and treatments in psychiatry, neurology, cancer and inflammatory diseases, and many of them inhibit HDAC3 activity. Therefore it is important to have a thorough understanding of different physiological functions of HDAC3. This knowledge will help predict and reduce the side effects of HDAC inhibitors, and will help uncover new applications for them.

In Chapter 3 and Chapter 4, I reported that REV-ERBs recruit HDAC3 to repress clock genes such as Bmal1 and Npas2. Do REV-ERBs recruit HDAC3 to repress clock genes in the central clock and other peripheral clocks? Is HDAC3 required for normal circadian rhythms? To answer the first question, I will examine HDAC3 recruitment at the Bmal1 and Npas2 genes in the SCN and other peripheral tissues, such as fat and muscle. To determine if HDAC3 is required for normal circadian rhythms, I will cross the HDAC3$^{fl/fl}$ mice to Rosa26-Cre-ER mice (these mice display tamoxifen-inducible Cre activity in all tissue types), and induce HDAC3 deletion in adult mice. We can then assess the circadian rhythms in these mice by measuring clock gene expression in the SCN and many peripheral tissues, such as liver, muscle, and fat, and by measuring food intake and locomoter activity in regular light dark cycles and in constant darkness. An alternative is to delete HDAC3 in mouse embryonic fibroblasts and measure the oscillation of clock genes after serum shock.

In Chapter 3, I reported a surprising overlapping between REV-ERB and HDAC3 genomic binding, and showed that recruitment of HDAC3 at many sites are REV-ERB dependent. But it is unlikely that HDAC3 is completely 'enslaved' by REV-ERBs in other tissues. Indeed, HDAC3 may not even be recruited by nuclear receptors, as shown in macrophages (Mullican et al., 2011). In the future, I hope to explore HDAC3 functions in other tissues and understand how HDAC3 is recruited in these tissues. I will focus on metabolic organs, such as muscle, heart and adipose tissues, and begin with HDAC3 ChIP-seq. ChIP-seq may reveal HDAC3 targets that account for the metabolic disorders

induced by HDAC3 deletion in these tissues (Sun et al., 2011), and motif analysis of

HDAC3 cistromes helps identify transcription factors that mediate HDAC3 recruitment.
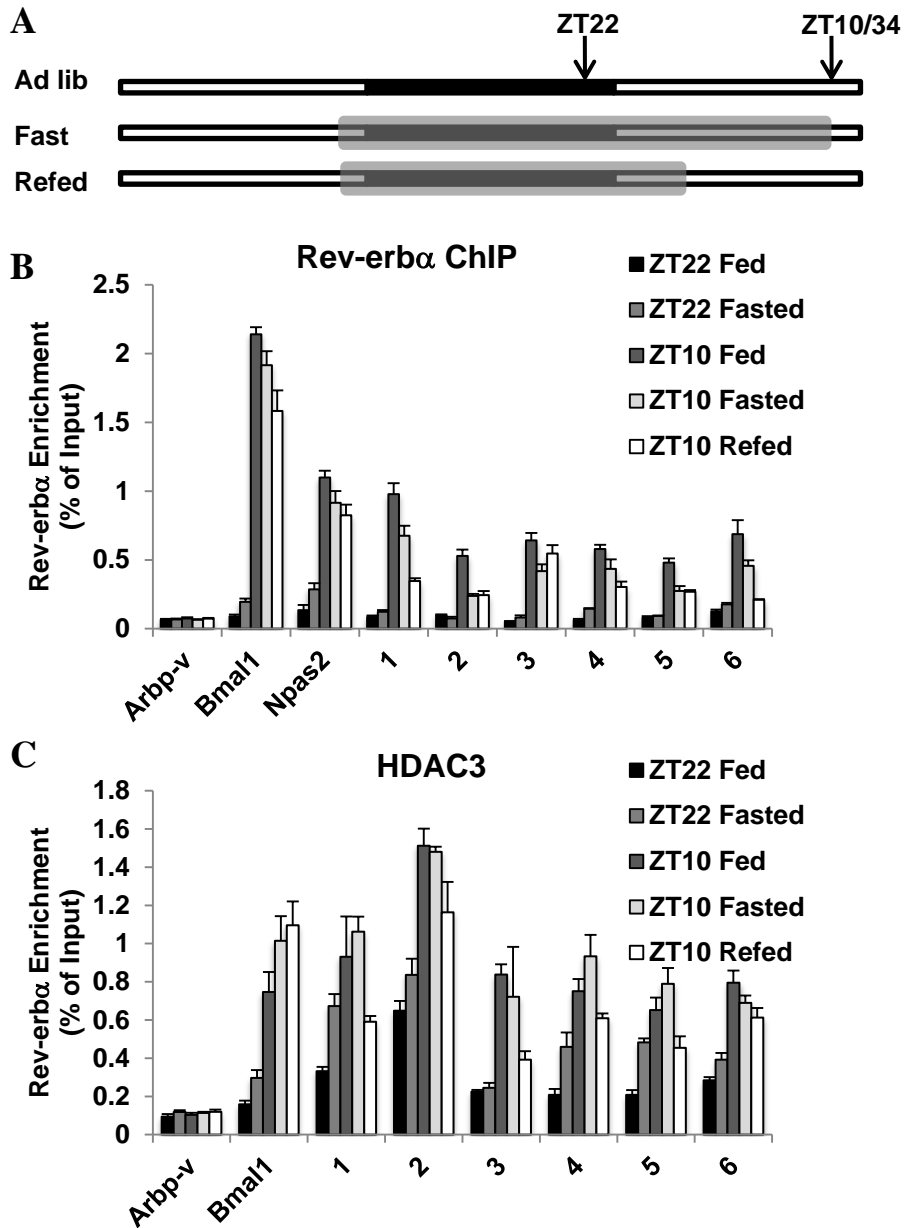
Figure 5.1 REV-ERBα and HDAC3 Recruitment in Response to Food Intake.

A. Wild type were fasted and refed as indicated in regular light/dark cycles. The gray area indicates the period without food. Liver tissues were collected at ZT22 and ZT10/34 as indicated by the arrow.

B. C. REV-ERBα (B) and HDAC3 (C) recruitment in livers collected in A were interrogated by ChIP-PCR. Values are mean ± s.e.m (n=4-5).
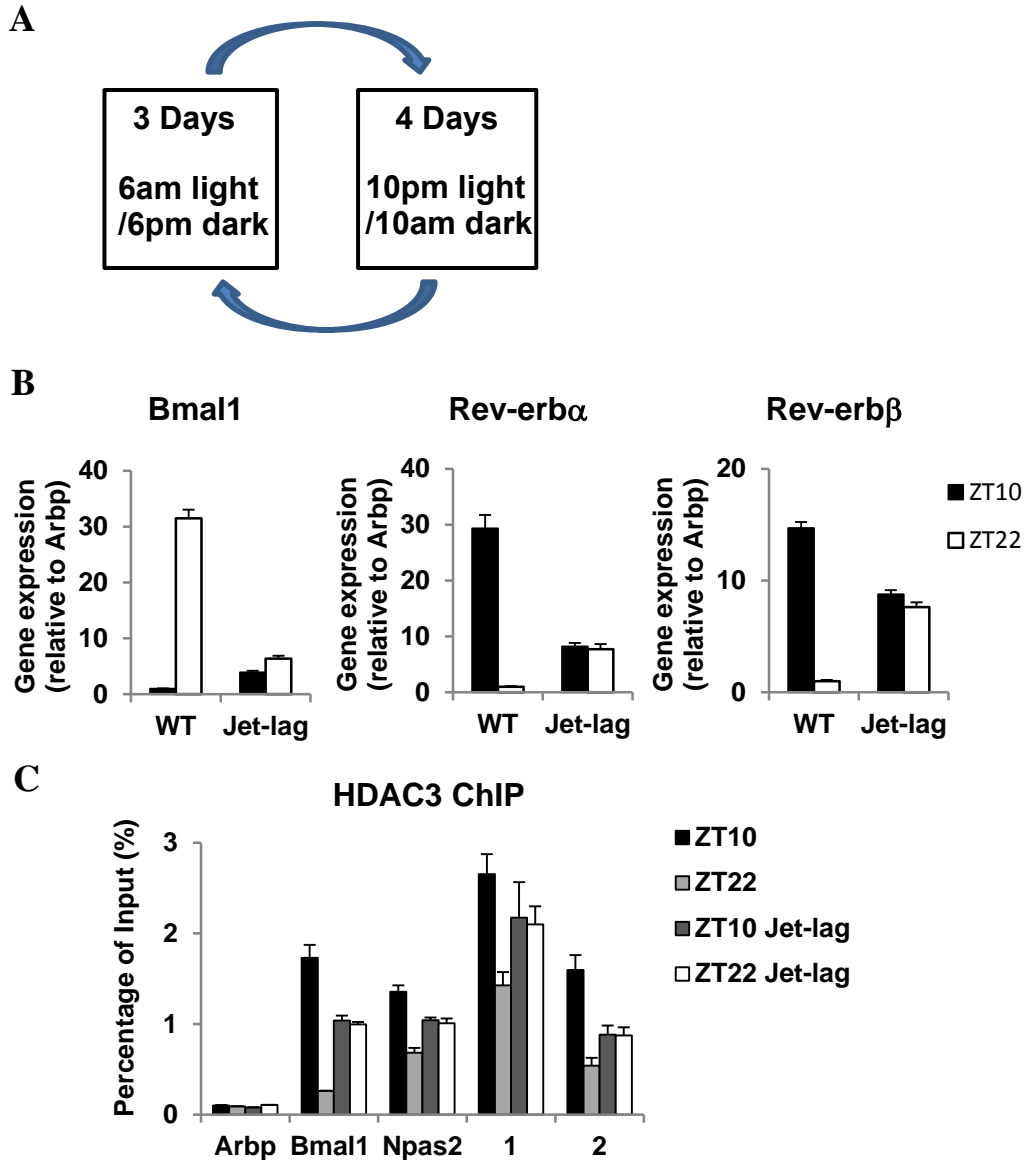
A. The chronic jet-lag treatment. Each week, the light/dark onset time was advanced by 8 hours; 3 days later, it was switched back until next week.

B. Bmal1, Rev-erbα and Rev-erbβ expression was measured in livers harvested at ZT10 and ZT22 from 14 week old jet-lag mice or mice housed under regulator light/dark cycles (WT). Values are mean ± s.e.m (n=3).

C. HDAC3 (C) recruitment in livers harvested at ZT10 and ZT22 from 14 week old jet-lag mice mice housed under regulator light/dark cycles (WT). Values are mean ± s.e.m (n=3).
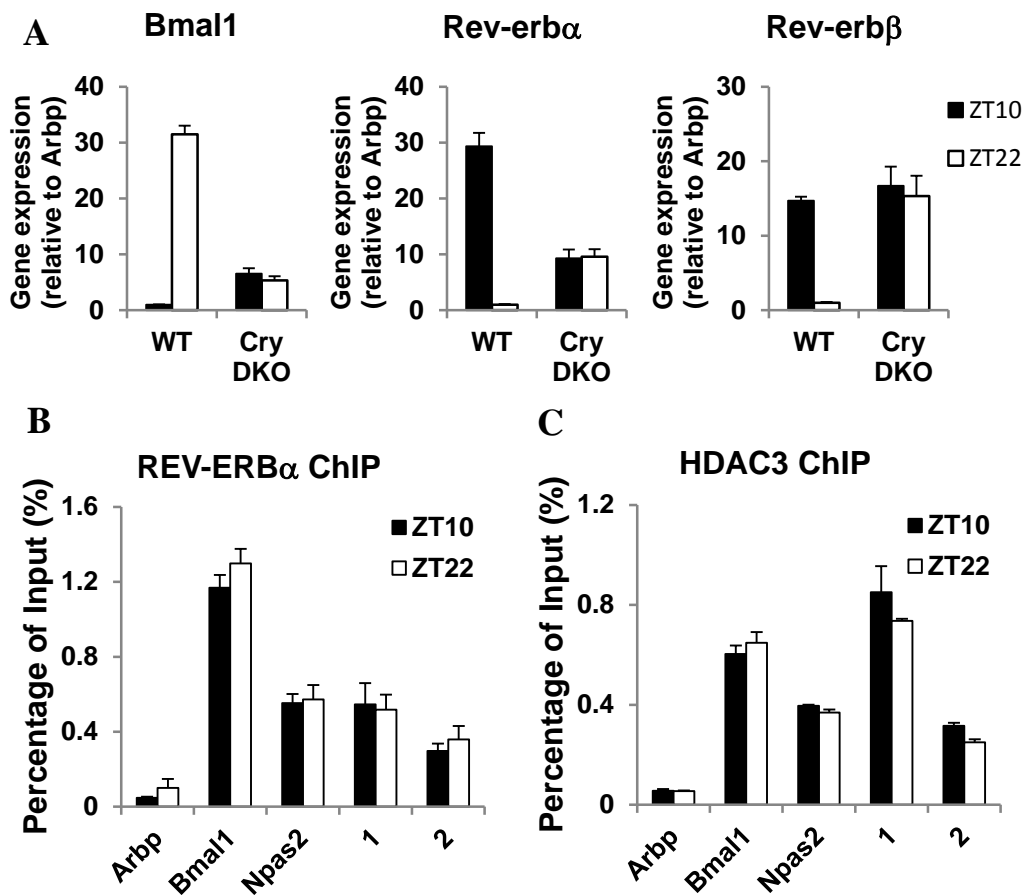
Figure 5.3 Loss of HDAC3 Binding Rhythm in Cry DKO Mice.

A. Bmal1, Rev-erbα and Rev-erbβ expression was measured in livers harvested at ZT10 and ZT22 from 14 week old Cry DKO mice. Values are mean ± s.e.m (n=3).

B. C. Rev-erbα (B) and HDAC3 (C) recruitment in livers harvested at ZT10 and ZT22 from 14 week old Cry DKO mice. Values are mean ± s.e.m (n=3).

REFERENCE

Adelmant, G., Bègue, A., Stéhelin, D., and Laudet, V. (1996). A functional Rev-erb alpha responsive element located in the human Rev-erb alpha promoter mediates a repressing activity. Proc. Natl. Acad. Sci. U.S.A. *93*, 3553–3558.

Akashi, M., and Takumi, T. (2005). The orphan nuclear receptor ROR[alpha] regulates circadian transcription of the mammalian core-clock Bmal1. Nat Struct Mol Biol *12*, 441–448.

Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H., and Kyriacou, C.P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic Nucleus. Current Biology *12*, 540–550.

Alenghat, T., Meyers, K., Mullican, S.E., Leitner, K., Adeniji-Adele, A., Avila, J., Bućan, M., Ahima, R.S., Kaestner, K.H., and Lazar, M.A. (2008). Nuclear receptor corepressor-histone deacetylase 3 governs circadian metabolic physiology. Nature *456*, 997–1000.

André, E., Conquet, F., Steinmayr, M., Stratton, S.C., Porciatti, V., and Becker-André, M. (1998). Disruption of retinoid-related orphan receptor beta changes circadian behavior, causes retinal degeneration and leads to vacillans phenotype in mice. EMBO J. *17*, 3867–3877.

Antunes, L.C., Levandovski, R., Dantas, G., Caumo, W., and Hidalgo, M.P. (2010). Obesity and shift work: chronobiological aspects. Nutrition Research Reviews *23*, 155–168.

Anzulovich, A., Mir, A., Brewer, M., Ferreyra, G., Vinson, C., and Baler, R. (2006). Elovl3: a model gene to dissect homeostatic links between the circadian clock and nutritional status. J. Lipid Res. *47*, 2690–2700.

Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., Mostoslavsky, R., Alt, F.W., and Schibler, U. (2008). SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell *134*, 317–328.

Asher, G., Reinke, H., Altmeyer, M., Gutierrez-Arcelus, M., Hottiger, M.O., and Schibler, U. (2010). Poly(ADP-Ribose) Polymerase 1 participates in the phase entrainment of circadian clocks to feeding. Cell *142*, 943–953.

Back, P., Hamprecht, B., and Lynen, F. (1969). Regulation of cholesterol biosynthesis in rat liver: Diurnal changes of activity and influence of bile acids. Archives of Biochemistry and Biophysics *133*, 11–21.

De Bacquer, D., Van Risseghem, M., Clays, E., Kittel, F., De Backer, G., and Braeckman, L. (2009). Rotating shift work and the metabolic syndrome: a prospective study. Int J Epidemiol *38*, 848–854.

Bae, K., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., and Weaver, D.R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron *30*, 525–536.

Bain, D.L., Heneghan, A.F., Connaghan-Jones, K.D., and Miura, M.T. (2007). Nuclear receptor structure: implications for function. Annual Review of Physiology *69*, 201–220.

Balsalobre, A., Damiola, F., and Schibler, U. (1998). A Serum Shock Induces circadian gene expression in mammalian tissue culture cells. Cell *93*, 929–937.

Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schütz, G., and Schibler, U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science *289*, 2344–2347.

Bass, J., and Takahashi, J.S. (2010). Circadian integration of metabolism and energetics. Science *330*, 1349–1354.

Bhaskara, S., Chyla, B.J., Amann, J.M., Knutson, S.K., Cortez, D., Sun, Z.-W., and Hiebert, S.W. (2008). Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control. Mol. Cell *30*, 61–72.

Bledsoe, R.K., Montana, V.G., Stanley, T.B., Delves, C.J., Apolito, C.J., McKee, D.D., Consler, T.G., Parks, D.J., Stewart, E.L., Willson, T.M., et al. (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor Dimerization and Coactivator Recognition. Cell *110*, 93–105.

Boergesen, M., Pedersen, T.A., Gross, B., Van Heeringen, S.J., Hagenbeek, D., Bindesbøll, C., Caron, S., Lalloyer, F., Steffensen, K.R., Nebb, H.I., Gustafsson, J.A., Stunnenberg, H.G., Staels, B., and Mandrup, S. (2012). Genome-wide profiling of liver X receptor, retinoid X receptor, and peroxisome proliferator-activated receptor α in mouse liver reveals extensive sharing of binding sites. Mol Cell Biol *32*, 852–867.

Bonnelye, E., Vanacker, J.M., Desbiens, X., Begue, A., Stehelin, D., and Laudet, V. (1994). Rev-erb beta, a new member of the nuclear receptor superfamily, is expressed in the nervous system during chicken development. Cell Growth Differ. *5*, 1357–1365.

Brown, S.A., Zumbrunn, G., Fleury-Olela, F., Preitner, N., and Schibler, U. (2002). Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Current Biology *12*, 1574–1583.

Brown, S.A., Ripperger, J., Kadener, S., Fleury-Olela, F., Vilbois, F., Rosbash, M., and Schibler, U. (2005). PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. Science *308*, 693–696.

Brunt, E.M. (2010). Pathology of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol *7*, 195–203.

Bugge, A., Feng, D., Everett, L.J., Briggs, E.R., Mullican, S.E., Wang, F., Jager, J., and Lazar, M.A. (2012). Rev-Erbα and Rev-Erbβ coordinately protect the circadian clock and normal metabolic function. Genes Dev. *26*, 657–667.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., and Bradfield, C.A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009–1017.

Canaple, L., Rambaud, J., Dkhissi-Benyahya, O., Rayet, B., Tan, N.S., Michalik, L., Delaunay, F., Wahli, W., and Laudet, V. (2006). Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock. Mol. Endocrinol. *20*, 1715–1727.

Cantó, C., and Auwerx, J. (2011). Calorie Restriction: Is AMPK a key sensor and effector? Physiology *26*, 214–224.

Cantó, C., Gerhart-Hines, Z., Feige, J.N., Lagouge, M., Noriega, L., Milne, J.C., Elliott, P.J., Puigserver, P., and Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature *458*, 1056–1060.

Carlberg, C., Hooft van Huijsduijnen, R., Staple, J.K., DeLamarter, J.F., and Becker-André, M. (1994). RZRs, a new family of retinoid-related orphan receptors that function as both monomers and homodimers. Mol. Endocrinol. *8*, 757–770.

Cermakian, N., Monaco, L., Pando, M.P., Dierich, A., and Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. EMBO J *20*, 3967–3974.

Charmandari, E., Chrousos, G.P., Lambrou, G.I., Pavlaki, A., Koide, H., Ng, S.S.M., and Kino, T. (2011). Peripheral CLOCK regulates target-tissue glucocorticoid receptor transcriptional activity in a circadian fashion in man. PLoS One *6*.

Chauvet, C., Bois-Joyeux, B., and Danan, J.-L. (2002). Retinoic acid receptor-related orphan receptor (ROR) alpha4 is the predominant isoform of the nuclear receptor RORalpha in the liver and is up-regulated by hypoxia in HepG2 human hepatoma cells. Biochem J *364*, 449–456.

Chawla, A., and Lazar, M.A. (1993). Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation. J. Biol. Chem. *268*, 16265–16269.

Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W., Jiang, J., et al. (2008). Integration of external signaling pathways with the core transcriptional netwok in embryonic stem cells. Cell *133*, 1106–1117.

Cho, H., Zhao, X., Hatori, M., Yu, R.T., Barish, G.D., Lam, M.T., Chong, L.-W., DiTacchio, L., Atkins, A.R., Glass, C.K., et al. (2012). Regulation of circadian behaviour and metabolism by REV-ERBα and REV-ERBβ. Nature *485, 123-127*

Chomez, P., Neveu, I., Mansén, A., Kiesler, E., Larsson, L., Vennström, B., and Arenas, E. (2000). Increased cell death and delayed development in the cerebellum of mice lacking the rev-erbA(alpha) orphan receptor. Development *127*, 1489–1498.

Chopin-Delannoy, S., Thénot, S., Delaunay, F., Buisine, E., Begue, A., Duterque-Coquillaud, M., and Laudet, V. (2003). A specific and unusual nuclear localization signal in the DNA binding domain of the Rev-erb orphan receptors. J. Mol. Endocrinol. *30*, 197–211.

Chopra, A.R., Louet, J.-F., Saha, P., An, J., DeMayo, F., Xu, J., York, B., Karpen, S., Finegold, M., Moore, D., et al. (2008). Absence of the SRC-2 coactivator results in a glycogenopathy resembling Von Gierke's Disease. Science *322*, 1395–1399.

Christensen, B.C., and Marsit, C.J. (2011). Epigenomics in environmental health. Front Genet *2*.

Crumbley, C., Wang, Y., Kojetin, D.J., and Burris, T.P. (2010). Characterization of the core mammalian clock component, NPAS2, as a REV-ERBα/RORα target gene. J. Biol. Chem. *285*, 35386–35392.

Curtis, A.M., Seo, S., Westgate, E.J., Rudic, R.D., Smyth, E.M., Chakravarti, D., FitzGerald, G.A., and McNamara, P. (2004). Histone acetyltransferase-dependent chromatin remodeling and the vascular clock. J. Biol. Chem. *279*, 7091–7097.

Dallmann, R., DeBruyne, J.P., and Weaver, D.R. (2011). Photic resetting and entrainment in CLOCK-deficient mice. J. Biol. Rhythms *26*, 390–401.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev *14*, 2950–2961.

DeBruyne, J.P., Noton, E., Lambert, C.M., Maywood, E.S., Weaver, D.R., and Reppert, S.M. (2006). A Clock Shock: Mouse CLOCK is not required for circadian oscillator function. Neuron *50*, 465–477.

DeBruyne, J.P., Weaver, D.R., and Reppert, S.M. (2007). CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nat Neurosci *10*, 543–545.

Dibner, C., Schibler, U., and Albrecht, U. (2010). The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annual Review of Physiology *72*, 517–549.

Dioum, E.M., Rutter, J., Tuckerman, J.R., Gonzalez, G., Gilles-Gonzalez, M.-A., and McKnight, S.L. (2002). NPAS2: A gas-responsive transcription factor. Science *298*, 2385–2387.

DiTacchio, L., Le, H.D., Vollmers, C., Hatori, M., Witcher, M., Secombe, J., and Panda, S. (2011). Histone lysine demethylase JARID1a activates CLOCK-BMAL1 and influences the circadian clock. Science *333*, 1881–1885.

Doi, M., Hirayama, J., and Sassone-Corsi, P. (2006). Circadian regulator CLOCK is a histone acetyltransferase. Cell *125*, 497–508.

Downes, M., Carozzi, A.J., and Muscat, G.E. (1995). Constitutive expression of the orphan receptor, Rev-erbA alpha, inhibits muscle differentiation and abrogates the expression of the myoD gene family. Mol. Endocrinol. *9*, 1666–1678.

Dudley, C.A., Erbel-Sieler, C., Estill, S.J., Reick, M., Franken, P., Pitts, S., and McKnight, S.L. (2003). Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. Science *301*, 379–383.

Duez, H., Van der Veen, J.N., Duhem, C., Pourcet, B., Touvier, T., Fontaine, C., Derudas, B., Baugé, E., Havinga, R., Bloks, V.W., et al. (2008). Regulation of bile acid synthesis by the nuclear receptor Rev-erbalpha. Gastroenterology *135*, 689–698.

Dufour, C.R., Levasseur, M.-P., Pham, N.H.H., Eichner, L.J., Wilson, B.J., Charest-Marcotte, A., Duguay, D., Poirier-Héon, J.-F., Cermakian, N., and Giguère, V. (2011). Genomic convergence among ERRα, PROX1, and BMAL1 in the control of metabolic clock outputs. PLoS Genet *7*.

Dumas, B., Harding, H.P., Choi, H.S., Lehmann, K.A., Chung, M., Lazar, M.A., and Moore, D.D. (1994). A new orphan member of the nuclear hormone receptor superfamily closely related to Rev-Erb. Mol. Endocrinol. *8*, 996–1005.

Duong, H.A., Robles, M.S., Knutti, D., and Weitz, C.J. (2011). A molecular mechanism for circadian clock negative feedback. Science *332*, 1436–1439.

Etchegaray, J.-P., Lee, C., Wade, P.A., and Reppert, S.M. (2002). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature *421*, 177–182.

Etchegaray, J.-P., Lee, C., Wade, P.A., and Reppert, S.M. (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature *421*, 177–182.

Etchegaray, J.-P., Yang, X., DeBruyne, J.P., Peters, A.H.F.M., Weaver, D.R., Jenuwein, T., and Reppert, S.M. (2006). The polycomb group protein EZH2 is required for mammalian circadian clock function. J. Biol. Chem. *281*, 21209–21215.

Farnham, P.J. (2009). Insights from genomic profiling of transcription factors. Nature Reviews Genetics *10*, 605–616.

Forman, B.M., Chen, J., Blumberg, B., Kliewer, S.A., Henshaw, R., Ong, E.S., and Evans, R.M. (1994). Cross-talk among ROR alpha 1 and the Rev-erb family of orphan nuclear receptors. Mol. Endocrinol. *8*, 1253–1261.

Froy, O. (2007). The relationship between nutrition and circadian rhythms in mammals. Front Neuroendocrinol *28*, 61–71.

Gallego, M., and Virshup, D.M. (2007). Post-translational modifications regulate the ticking of the circadian clock. Nature Reviews Molecular Cell Biology *8*, 139–148.

Gao, G.-P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J.M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. PNAS *99*, 11854–11859.

Gatfield, D., Le Martelot, G., Vejnar, C.E., Gerlach, D., Schaad, O., Fleury-Olela, F., Ruskeepää, A.-L., Oresic, M., Esau, C.C., Zdobnov, E.M., et al. (2009). Integration of microRNA miR-122 in hepatic circadian gene expression. Genes Dev *23*, 1313–1326.

Gerstein, M.B., Lu, Z.J., Nostrand, E.L.V., Cheng, C., Arshinoff, B.I., Liu, T., Yip, K.Y., Robilotto, R., Rechtsteiner, A., Ikegami, K., et al. (2010). Integrative Analysis of the Caenorhabditis elegans Genome by the modENCODE Project. Science *330*, 1775–1787.

Gery, S., Virk, R.K., Chumakov, K., Yu, A., and Koeffler, H.P. (2007). The clock gene Per2 links the circadian system to the estrogen receptor. Oncogene *26*, 7916–7920.

Giguère, V., Tini, M., Flock, G., Ong, E., Evans, R.M., and Otulakowski, G. (1994). Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors. Genes Dev. *8*, 538–553.

Gilles-Gonzalez, M.-A., and Gonzalez, G. (2004). Signal transduction by heme-containing PAS-domain proteins. J. Appl. Physiol. *96*, 774–783.

Gimble, J.M., and Floyd, Z.E. (2009). Fat circadian biology. J. Appl. Physiol. *107*, 1629–1637.

Goodson, M., Jonas, B.A., and Privalsky, M.A. (2005). Corepressors: custom tailoring and alterations while you wait. Nucl Recept Signal *3*.

Green, C.B., Takahashi, J.S., and Bass, J. (2008). The meter of metabolism. Cell *134*, 728–742.

Grimaldi, B., Bellet, M.M., Katada, S., Astarita, G., Hirayama, J., Amin, R.H., Granneman, J.G., Piomelli, D., Leff, T., and Sassone-Corsi, P. (2010). PER2 controls lipid metabolism by direct regulation of PPARγ. Cell Metab. *12*, 509–520.

Guenther, M.G., Lane, W.S., Fischle, W., Verdin, E., Lazar, M.A., and Shiekhattar, R. (2000). A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. Genes Dev. *14*, 1048–1057.

Guenther, M.G., Barak, O., and Lazar, M.A. (2001). The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol. Cell. Biol. *21*, 6091–6101.

Guillaumond, F., Dardente, H., Giguère, V., and Cermakian, N. (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J. Biol. Rhythms *20*, 391–403.

Gupta, N., and Ragsdale, S.W. (2011). Thiol-disulfide redox dependence of heme binding and heme ligand switching in nuclear hormone receptor rev-erbβ. J. Biol. Chem. *286*, 4392–4403.

Harding, H.P., and Lazar, M.A. (1993). The orphan receptor Rev-ErbA alpha activates transcription via a novel response element. Mol. Cell. Biol. *13*, 3113–3121.

He, A., Kong, S.W., Ma, Q., and Pu, W.T. (2011). Co-occupancy by multiple cardiac transcription factors identifies transcriptional enhancers active in heart. PNAS.

He, Y.W., Deftos, M.L., Ojala, E.W., and Bevan, M.J. (1998). RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. Immunity *9*, 797–806.

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., and Glass, C.K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol. Cell *38*, 576–589.

Hems, D.A., Rath, E.A., and Verrinder, T.R. (1975). Fatty acid synthesis in liver and adipose tissue of normal and genetically obese (ob/ob) mice during the 24-hour cycle. Biochem J *150*, 167–173.

Hermanson, O., Glass, C.K., and Rosenfeld, M.G. (2002). Nuclear receptor coregulators: multiple modes of modification. Trends in Endocrinology & Metabolism *13*, 55–60.

Higgins, M., Kawachi, T., and Rudney, H. (1971). The mechanism of the diurnal variation of hepatic HMG-CoA reductase activity in the rat. Biochemical and Biophysical Research Communications *45*, 138–144.

Hirayama, J., Sahar, S., Grimaldi, B., Tamaru, T., Takamatsu, K., Nakahata, Y., and Sassone-Corsi, P. (2007). CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature *450*, 1086–1090.

Van der Horst, G.T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., De Wit, J., Verkerk, A., Eker, A.P., Van Leenen, D., et al. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature *398*, 627–630.

Hu, X., and Lazar, M.A. (2000). Transcriptional repression by nuclear hormone receptors. Trends Endocrinol. Metab. *11*, 6–10.

Hu, X., Li, Y., and Lazar, M.A. (2001). Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. Mol. Cell. Biol. *21*, 1747–1758.

Huang, W., Ramsey, K.M., Marcheva, B., and Bass, J. (2011). Circadian rhythms, sleep, and metabolism. J Clin Invest *121*, 2133–2141.

Hughes, M.E., DiTacchio, L., Hayes, K.R., Vollmers, C., Pulivarthy, S., Baggs, J.E., Panda, S., and Hogenesch, J.B. (2009). Harmonics of circadian gene transcription in mammals. PLoS Genet *5*, e1000442.

Ishizuka, T., and Lazar, M.A. (2003). The N-CoR/Histone deacetylase 3 complex is required for repression by thyroid hormone receptor. Mol Cell Biol *23*, 5122–5131.

Jetten, A.M. (2009). Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. Nucl Recept Signal *7*.

Jetten, A.M., Kang, H.S., and Takeda, Y. (2013). Retinoic acid-related orphan receptors α and γ: key regulators of lipid/glucose metabolism, inflammation, and insulin sensitivity. Front Endocrinol (Lausanne) *4*.

Jin, L., Martynowski, D., Zheng, S., Wada, T., Xie, W., and Li, Y. (2010). Structural basis for hydroxycholesterols as natural ligands of orphan nuclear receptor RORgamma. Mol. Endocrinol. *24*, 923–929.

Kaasik, K., and Lee, C.C. (2004). Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature *430*, 467–471.

Kang, H.S., Angers, M., Beak, J.Y., Wu, X., Gimble, J.M., Wada, T., Xie, W., Collins, J.B., Grissom, S.F., and Jetten, A.M. (2007). Gene expression profiling reveals a regulatory role for RORα and RORγ in phase I and phase II metabolism. Physiol. Genomics *31*, 281–294.

Kassam, A., Capone, J.P., and Rachubinski, R.A. (1999). Orphan nuclear hormone receptor RevErbalpha modulates expression from the promoter of the hydratase-dehydrogenase gene by inhibiting peroxisome proliferator-activated receptor alpha-dependent transactivation. J. Biol. Chem. *274*, 22895–22900.

Katada, S., and Sassone-Corsi, P. (2010). The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. Nature Structural & Molecular Biology *17*, 1414–1421.

Kawamoto, T., Noshiro, M., Furukawa, M., Honda, K.K., Nakashima, A., Ueshima, T., Usui, E., Katsura, Y., Fujimoto, K., Honma, S., et al. (2006). Effects of fasting and re-feeding on the expression of Dec1, Per1, and other clock-related genes. J Biochem *140*, 401–408.

Kida, K., Nishio, T., Yokozawa, T., Nagai, K., Matsuda, H., and Nakagawa, H. (1980). The circadian change of gluconeogenesis in the liver in vivo in fed rats. J. Biochem. *88*, 1009–1013.

Kimura, A., Matsubara, K., and Horikoshi, M. (2005). A decade of histone acetylation: marking eukaryotic chromosomes with specific codes. J Biochem *138*, 647–662.

Knutson, S.K., Chyla, B.J., Amann, J.M., Bhaskara, S., Huppert, S.S., and Hiebert, S.W. (2008). Liver-specific deletion of histone deacetylase 3 disrupts metabolic transcriptional networks. EMBO J. *27*, 1017–1028.

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., and Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metabolism *6*, 414–421.

Kornmann, B., Schaad, O., Bujard, H., Takahashi, J.S., and Schibler, U. (2007). System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol *5*.

Laitinen, S., Fontaine, C., Fruchart, J.C., and Staels, B. (2005). The role of the orphan nuclear receptor Rev-Erb alpha in adipocyte differentiation and function. Biochimie *87*, 21–25.

Lamia, K.A., Storch, K.-F., and Weitz, C.J. (2008). Physiological significance of a peripheral tissue circadian clock. PNAS *105*, 15172–15177.

Lamia, K.A., Sachdeva, U.M., DiTacchio, L., Williams, E.C., Alvarez, J.G., Egan, D.F., Vasquez, D.S., Juguilon, H., Panda, S., Shaw, R.J., et al. (2009). AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science *326*, 437–440.

Lamia, K.A., Papp, S.J., Ruth, T.Y., Barish, G.D., Uhlenhaut, N.H., Jonker, J.W., Downes, M., and Evans, R.M. (2011). Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature *480*, 552–556.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. *10*, R25.

Lau, P., Bailey, P., Dowhan, D.H., and Muscat, G.E. (1999). Exogenous expression of a dominant negative RORalpha1 vector in muscle cells impairs differentiation: RORalpha1 directly interacts with p300 and myoD. Nucleic Acids Res *27*, 411–420.

Lau, P., Fitzsimmons, R.L., Raichur, S., Wang, S.-C.M., Lechtken, A., and Muscat, G.E.O. (2008). The orphan nuclear receptor, RORalpha, regulates gene expression that controls lipid metabolism: staggerer (SG/SG) mice are resistant to diet-induced obesity. J. Biol. Chem. *283*, 18411–18421.

Lazar, M.A. (2003). Nuclear receptor corepressors. Nucl Recept Signal *1*.

Lazar, M.A., Hodin, R.A., Darling, D.S., and Chin, W.W. (1989). A novel member of the thyroid/steroid hormone receptor family is encoded by the opposite strand of the rat c-erbA alpha transcriptional unit. Mol. Cell. Biol. *9*, 1128–1136.

Leavens, K.F., Easton, R.M., Shulman, G.I., Previs, S.F., and Birnbaum, M.J. (2009). Akt2 is required for hepatic lipid accumulation in models of insulin resistance. Cell Metab. *10*, 405–418.

Levi, F., and Schibler, U. (2007). Circadian Rhythms: Mechanisms and therapeutic implications. Annual Review of Pharmacology and Toxicology *47*, 593–628.

Li, J., Wang, J., Wang, J., Nawaz, Z., Liu, J.M., Qin, J., and Wong, J. (2000). Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J. *19*, 4342–4350.

Li, Y., Lambert, M.H., and Xu, H.E. (2003). Activation of nuclear receptors: a perspective from structural genomics. Structure *11*, 741–746.

Liu, A.C., Tran, H.G., Zhang, E.E., Priest, A.A., Welsh, D.K., and Kay, S.A. (2008a). Redundant function of REV-ERBα and β and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. PLoS Genet *4*, e1000023.

Liu, X.S., Brutlag, D.L., and Liu, J.S. (2002). An algorithm for finding protein-DNA binding sites with applications to chromatin-immunoprecipitation microarray experiments. Nat. Biotechnol. *20*, 835–839.

Liu, Y., Dentin, R., Chen, D., Hedrick, S., Ravnskjaer, K., Schenk, S., Milne, J., Meyers, D.J., Cole, P., Yates, J., et al. (2008b). A fasting inducible switch modulates gluconeogenesis via activator-coactivator exchange. Nature *456*, 269–273.

Ma, K., Xiao, R., Tseng, H.-T., Shan, L., Fu, L., and Moore, D.D. (2009). Circadian dysregulation disrupts bile acid homeostasis. PLoS ONE *4*, e6843.

Marcheva, B., Ramsey, K.M., Buhr, E.D., Kobayashi, Y., Su, H., Ko, C.H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M.H., et al. (2010). Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. Nature *466*, 627–631.

Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B., Sasso, G.L., Moschetta, A., and Schibler, U. (2009). REV-ERBα participates in circadian SREBP signaling and bile acid homeostasis. PLoS Biol *7*.

Maywood, E.S., O'Neill, J.S., Reddy, A.B., Chesham, J.E., Prosser, H.M., Kyriacou, C.P., Godinho, S.I.H., Nolan, P.M., and Hastings, M.H. (2007). Genetic and molecular analysis of the central and peripheral circadian clockwork of mice. Cold Spring Harb. Symp. Quant. Biol. *72*, 85–94.

McCarthy, J.J., Andrews, J.L., McDearmon, E.L., Campbell, K.S., Barber, B.K., Miller, B.H., Walker, J.R., Hogenesch, J.B., Takahashi, J.S., and Esser, K.A. (2007).

Identification of the circadian transcriptome in adult mouse skeletal muscle. Physiological Genomics *31*, 86–95.

Medvedev, A., Yan, Z.H., Hirose, T., Giguère, V., and Jetten, A.M. (1996). Cloning of a cDNA encoding the murine orphan receptor RZR/ROR gamma and characterization of its response element. Gene *181*, 199–206.

Miyajima, N., Kadowaki, Y., Fukushige, S., Shimizu, S., Semba, K., Yamanashi, Y., Matsubara, K., Toyoshima, K., and Yamamoto, T. (1988). Identification of two novel members of erbA superfamily by molecular cloning: the gene products of the two are highly related to each other. Nucleic Acids Res. *16*, 11057–11074.

Mongrain, V., Ruan, X., Dardente, H., Fortier, E.E., and Cermakian, N. (2008). Clock-dependent and independent transcriptional control of the two isoforms from the mouse Rorgamma gene. Genes Cells *13*, 1197–1210.

Mullican, S.E., Gaddis, C.A., Alenghat, T., Nair, M.G., Giacomin, P.R., Everett, L.J., Feng, D., Steger, D.J., Schug, J., Artis, D., et al. (2011). Histone deacetylase 3 Is an epigenomic brake in macrophage alternative activation. Genes Dev. *25*, 2480–2488.

Nader, N., Chrousos, G.P., and Kino, T. (2009). Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. FASEB J. *23*, 1572–1583.

Nakahata, Y., Kaluzova, M., Grimaldi, B., Sahar, S., Hirayama, J., Chen, D., Guarente, L.P., and Sassone-Corsi, P. (2008). The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell *134*, 329–340.

Nakahata, Y., Sahar, S., Astarita, G., Kaluzova, M., and Sassone-Corsi, P. (2009). Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. Science *324*, 654–657.

Naruse, Y., Oh-hashi, K., Iijima, N., Naruse, M., Yoshioka, H., and Tanaka, M. (2004). Circadian and light-induced transcription of clock gene Per1 depends on histone acetylation and deacetylation. Mol Cell Biol *24*, 6278–6287.

Nègre, N., Brown, C.D., Ma, L., Bristow, C.A., Miller, S.W., Wagner, U., Kheradpour, P., Eaton, M.L., Loriaux, P., Sealfon, R., et al. (2011). A cis-regulatory map of the Drosophila genome. Nature *471*, 527–531.

O'Neill, J.S., and Reddy, A.B. (2011). Circadian clocks in human red blood cells. Nature *469*, 498–503.

O'Neill, J.S., Ooijen, G. van, Dixon, L.E., Troein, C., Corellou, F., Bouget, F.-Y., Reddy, A.B., and Millar, A.J. (2011). Circadian rhythms persist without transcription in a eukaryote. Nature *469*, 554–558.

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M.W., and Eichele, G. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metabolism *4*, 163–173.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., and Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*, 307–320.

Pardee, K.I., Xu, X., Reinking, J., Schuetz, A., Dong, A., Liu, S., Zhang, R., Tiefenbach, J., Lajoie, G., Plotnikov, A.N., et al. (2009). The structural basis of gas-responsive transcription by the human nuclear hormone receptor REV-ERBbeta. PLoS Biol. *7*, e43.

Perissi, V., Jepsen, K., Glass, C.K., and Rosenfeld, M.G. (2010). Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet *11*, 109–123.

Phelan, C.A., Gampe, R.T., Jr, Lambert, M.H., Parks, D.J., Montana, V., Bynum, J., Broderick, T.M., Hu, X., Williams, S.P., Nolte, R.T., et al. (2010). Structure of Rev-erbalpha bound to N-CoR reveals a unique mechanism of nuclear receptor-co-repressor interaction. Nat. Struct. Mol. Biol. *17*, 808–814.

Pietroiusti, A., Neri, A., Somma, G., Coppeta, L., Iavicoli, I., Bergamaschi, A., and Magrini, A. (2010). Incidence of metabolic syndrome among night-shift healthcare workers. Occup Environ Med *67*, 54–57.

Portela, A., and Esteller, M. (2010). Epigenetic modifications and human disease. Nature Biotechnology *28*, 1057–1068.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell *110*, 251–260.

Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P., and Rastinejad, F. (2007). Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta. Nat. Struct. Mol. Biol. *14*, 1207–1213.

Ramakrishnan, S.N., Lau, P., Burke, L.J., and Muscat, G.E.O. (2005). Rev-erbbeta regulates the expression of genes involved in lipid absorption in skeletal muscle cells: evidence for cross-talk between orphan nuclear receptors and myokines. J. Biol. Chem. *280*, 8651–8659.

Ramsey, K.M., and Bass, J. (2011). Circadian clocks in fuel harvesting and energy homeostasis. Cold Spring Harb. Symp. Quant. Biol.

Ramsey, K.M., Yoshino, J., Brace, C.S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H.-K., Chong, J.L., Buhr, E.D., Lee, C., et al. (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. Science *324*, 651–654.

Raspé, E., Duez, H., Gervois, P., Fiévet, C., Fruchart, J.C., Besnard, S., Mariani, J., Tedgui, A., and Staels, B. (2001). Transcriptional regulation of apolipoprotein C-III gene expression by the orphan nuclear receptor RORalpha. J. Biol. Chem. *276*, 2865–2871.

Raspé, E., Duez, H., Mansén, A., Fontaine, C., Fiévet, C., Fruchart, J.-C., Vennström, B., and Staels, B. (2002). Identification of Rev-erbα as a physiological repressor of apoC-III gene transcription. J. Lipid Res. *43*, 2172–2179.

Reddy, A.B., Karp, N.A., Maywood, E.S., Sage, E.A., Deery, M., O'Neill, J.S., Wong, G.K.Y., Chesham, J., Odell, M., Lilley, K.S., et al. (2006). Circadian orchestration of the hepatic proteome. Current Biology *16*, 1107–1115.

Reddy, A.B., Maywood, E.S., Karp, N.A., King, V.M., Inoue, Y., Gonzalez, F.J., Lilley, K.S., Kyriacou, C.P., and Hastings, M.H. (2007). Glucocorticoid signaling synchronizes the liver circadian transcriptome. Hepatology *45*, 1478–1488.

Renaud, J.P., Harris, J.M., Downes, M., Burke, L.J., and Muscat, G.E. (2000). Structure-function analysis of the Rev-erbA and RVR ligand-binding domains reveals a large hydrophobic surface that mediates corepressor binding and a ligand cavity occupied by side chains. Mol. Endocrinol. *14*, 700–717.

Retnakaran, R., Flock, G., and Giguère, V. (1994). Identification of RVR, a novel orphan nuclear receptor that acts as a negative transcriptional regulator. Mol. Endocrinol. *8*, 1234–1244.

Rey, G., Cesbron, F., Rougemont, J., Reinke, H., Brunner, M., and Naef, F. (2011). Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. PLoS Biol *9*.

Ripperger, J.A., and Schibler, U. (2006). Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nature Genetics *38*, 369–374.

Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., and Puigserver, P. (2005). Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. Nature *434*, 113–118.

Rudic, R.D., McNamara, P., Curtis, A.-M., Boston, R.C., Panda, S., Hogenesch, J.B., and FitzGerald, G.A. (2004). BMAL1 and CLOCK, Two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol *2*, e377.

Rutter, J., Reick, M., Wu, L.C., and McKnight, S.L. (2001). Regulation of Clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science *293*, 510–514.

Sahar, S., Nin, V., Barbosa, M.T., Chini, E.N., and Sassone-Corsi, P. (2011). Altered behavioral and metabolic circadian rhythms in mice with disrupted NAD+ oscillation. Aging (Albany NY) *3*, 794–802.

Salmon-Divon, M., Dvinge, H., Tammoja, K., and Bertone, P. (2010). PeakAnalyzer: genome-wide annotation of chromatin binding and modification loci. BMC Bioinformatics *11*, 415.

Scheer, F.A.J.L., Hilton, M.F., Mantzoros, C.S., and Shea, S.A. (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc Natl Acad Sci U S A *106*, 4453–4458.

Schmutz, I., Ripperger, J.A., Baeriswyl-Aebischer, S., and Albrecht, U. (2010). The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors. Genes Dev *24*, 345–357.

Segraves, W.A., and Hogness, D.S. (1990). The E75 ecdysone-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily. Genes Dev. *4*, 204–219.

Shi, S., Hida, A., McGuinness, O.P., Wasserman, D.H., Yamazaki, S., and Johnson, C.H. (2010). Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. Curr. Biol. *20*, 316–321.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The Structural Basis of Estrogen Receptor/Coactivator Recognition and the Antagonism of This Interaction by Tamoxifen. Cell *95*, 927–937.

Shimba, S., Ishii, N., Ohta, Y., Ohno, T., Watabe, Y., Hayashi, M., Wada, T., Aoyagi, T., and Tezuka, M. (2005). Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. Proc. Natl. Acad. Sci. U.S.A. *102*, 12071–12076.

Shimba, S., Ogawa, T., Hitosugi, S., Ichihashi, Y., Nakadaira, Y., Kobayashi, M., Tezuka, M., Kosuge, Y., Ishige, K., Ito, Y., et al. (2011). Deficient of a clock gene, brain and muscle Arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation. PLoS ONE *6*, e25231.

Shin, H., Liu, T., Manrai, A.K., and Liu, X.S. (2009). CEAS: cis-regulatory element annotation system. Bioinformatics *25*, 2605–2606.

Siersbæk, R., Nielsen, R., John, S., Sung, M.-H., Baek, S., Loft, A., Hager, G.L., and Mandrup, S. (2011). Extensive chromatin remodelling and establishment of transcription factor "hotspots" during early adipogenesis. EMBO J. *30*, 1459–1472.

Solt, L.A., Wang, Y., Banerjee, S., Hughes, T., Kojetin, D.J., Lundasen, T., Shin, Y., Liu, J., Cameron, M.D., Noel, R., et al. (2012). Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. Nature *485*, 62–68.

Sonoda, J., Pei, L., and Evans, R.M. (2008). Nuclear receptors: Decoding metabolic disease. FEBS Letters *582*, 2–9.

Sookoian, S., Castaño, G., Gemma, C., Gianotti, T.-F., and Pirola, C.-J. (2007). Common genetic variations in CLOCK transcription factor are associated with nonalcoholic fatty liver disease. World J. Gastroenterol. *13*, 4242–4248.

Stokkan, K.-A., Yamazaki, S., Tei, H., Sakaki, Y., and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. Science *291*, 490–493.

Storch, K.-F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H., and Weitz, C.J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78–83.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41–45.

Sun, Z., Singh, N., Mullican, S.E., Everett, L.J., Li, L., Yuan, L., Liu, X., Epstein, J.A., and Lazar, M.A. (2011). Diet-induced lethality due to deletion of the Hdac3 gene in heart and skeletal muscle. J. Biol. Chem. *286*, 33301–33309.

Tahara, Y., Otsuka, M., Fuse, Y., Hirao, A., and Shibata, S. (2011). Refeeding after fasting elicits insulin-dependent regulation of Per2 and Rev-Erbα with shifts in the liver clock. J Biol Rhythms *26*, 230–240.

Takahata, S., Ozaki, T., Mimura, J., Kikuchi, Y., Sogawa, K., and Fujii-Kuriyama, Y. (2000). Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. Genes to Cells *5*, 739–747.

Takeda, Y., Kang, H.S., Angers, M., and Jetten, A.M. (2011). Retinoic acid-related orphan receptor γ directly regulates neuronal PAS domain protein 2 transcription in vivo. Nucleic Acids Res *39*, 4769–4782.

Takeda, Y., Jothi, R., Birault, V., and Jetten, A.M. (2012). RORγ directly regulates the circadian expression of clock genes and downstream targets in vivo. Nucleic Acids Res. *40*, 8519–8535.

Tao, W., Chen, S., Shi, G., Guo, J., Xu, Y., and Liu, C. (2011). SWItch/sucrose nonfermentable (SWI/SNF) complex subunit BAF60a integrates hepatic circadian clock and energy metabolism. Hepatology *54*, 1410–1420.

Thomas, P.D., Campbell, M.J., Kejariwal, A., Mi, H., Karlak, B., Daverman, R., Diemer, K., Muruganujan, A., and Narechania, A. (2003). PANTHER: a library of protein families and subfamilies indexed by function. Genome Res. *13*, 2129–2141.

Triqueneaux, G., Thenot, S., Kakizawa, T., Antoch, M.P., Safi, R., Takahashi, J.S., Delaunay, F., and Laudet, V. (2004). The orphan receptor Rev-erbalpha gene is a target of the circadian clock pacemaker. J. Mol. Endocrinol. *33*, 585–608.

Turek, F.W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D.R., et al. (2005). Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice. Science *308*, 1043–1045.

Ueda, H.R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S., et al. (2002). A transcription factor response element for gene expression during circadian night. Nature *418*, 534–539.

Um, J.H., Yang, S., Yamazaki, S., Kang, H., Viollet, B., Foretz, M., and Chung, J.H. (2007). Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2. J. Biol. Chem. *282*, 20794–20798.

Um, J.-H., Pendergast, J.S., Springer, D.A., Foretz, M., Viollet, B., Brown, A., Kim, M.K., Yamazaki, S., and Chung, J.H. (2011). AMPK regulates circadian rhythms in a tissue- and isoform-specific manner. PLoS ONE *6*, e18450.

Vieira, E., Nilsson, E.C., Nerstedt, A., Ormestad, M., Long, Y.C., Garcia-Roves, P.M., Zierath, J.R., and Mahlapuu, M. (2008). Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. *295*, E1032–1037.

Vitaterna, M.H., Selby, C.P., Todo, T., Niwa, H., Thompson, C., Fruechte, E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J., et al. (1999). Differential regulation of mammalian Period genes and circadian rhythmicity by cryptochromes 1 and 2. PNAS *96*, 12114–12119.

Vollmers, C., Gill, S., DiTacchio, L., Pulivarthy, S.R., Le, H.D., and Panda, S. (2009). Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. Proc Natl Acad Sci U S A *106*, 21453–21458.

Vu-Dac, N., Chopin-Delannoy, S., Gervois, P., Bonnelye, E., Martin, G., Fruchart, J.-C., Laudet, V., and Staels, B. (1998). The nuclear receptors peroxisome proliferator-activated receptor α and Rev-erbα mediate the species-specific regulation of apolipoprotein A-I expression by fibrates. J. Biol. Chem. *273*, 25713–25720.

Wang, J., and Lazar, M.A. (2008). Bifunctional role of Rev-erbα in adipocyte differentiation. Mol. Cell. Biol. *28*, 2213–2220.

Wang, J., Li, Y., Zhang, M., Liu, Z., Wu, C., Yuan, H., Li, Y.-Y., Zhao, X., and Lu, H. (2007). A zinc finger HIT domain-containing protein, ZNHIT-1, interacts with orphan nuclear hormone receptor Rev-erbbeta and removes Rev-erbbeta-induced inhibition of apoCIII transcription. FEBS J. *274*, 5370–5381.

Wang, J., Liu, N., Liu, Z., Li, Y., Song, C., Yuan, H., Li, Y., Zhao, X., and Lu, H. (2008). The orphan nuclear receptor Rev-erbβ recruits Tip60 and HDAC1 to regulate apolipoprotein CIII promoter. Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research *1783*, 224–236.

Wang, X.-S., Armstrong, M.E.G., Cairns, B.J., Key, T.J., and Travis, R.C. (2011). Shift work and chronic disease: the epidemiological evidence. Occup Med (Lond) *61*, 78–89.

Wang, Y., Kumar, N., Crumbley, C., Griffin, P.R., and Burris, T.P. (2010a). A second class of nuclear receptors for oxysterols: regulation of RORalpha and RORgamma activity by 24S-Hydroxycholesterol (Cerebrosterol). Biochim Biophys Acta *1801*, 917–923.

Wang, Y., Kumar, N., Solt, L.A., Richardson, T.I., Helvering, L.M., Crumbley, C., Garcia-Ordonez, R.D., Stayrook, K.R., Zhang, X., Novick, S., et al. (2010b). Modulation of retinoic acid receptor-related orphan receptor alpha and gamma activity by 7-oxygenated sterol ligands. J. Biol. Chem. *285*, 5013–5025.

Wang, Z., Zang, C., Cui, K., Schones, D.E., Barski, A., Peng, W., and Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell *138*, 1019–1031.

Waterhouse, J. (1999). Jet-lag and shift work: (1). Circadian rhythms. J R Soc Med *92*, 398–401.

Welsh, D.K., Takahashi, J.S., and Kay, S.A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. Annual Review of Physiology *72*, 551–577.

Witt, O., Deubzer, H.E., Milde, T., and Oehme, I. (2009). HDAC family: What are the cancer relevant targets? Cancer Letters *277*, 8–21.

Woo, E.-J., Jeong, D.G., Lim, M.-Y., Jun Kim, S., Kim, K.-J., Yoon, S.-M., Park, B.-C., and Ryu, S.E. (2007). Structural insight into the constitutive repression function of the nuclear receptor Rev-erbbeta. J. Mol. Biol. *373*, 735–744.

Wu, N., Yin, L., Hanniman, E.A., Joshi, S., and Lazar, M.A. (2009). Negative feedback maintenance of heme homeostasis by its receptor, Rev-erbα. Genes Dev. *23*, 2201–2209.

Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Straume, M., Mangelsdorf, D.J., and Evans, R.M. (2006). Nuclear receptor expression links the circadian clock to metabolism. Cell *126*, 801–810.

Ye, T., Krebs, A.R., Choukrallah, M.-A., Keime, C., Plewniak, F., Davidson, I., and Tora, L. (2011). seqMINER: an integrated ChIP-seq data interpretation platform. Nucleic Acids Res. *39*, e35.

Yin, L., and Lazar, M.A. (2005). The orphan nuclear receptor Rev-erbα recruits the N-CoR/Histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene. Molecular Endocrinology *19*, 1452–1459.

Yin, L., Wang, J., Klein, P.S., and Lazar, M.A. (2006). Nuclear receptor Rev-erbα is a critical lithium-sensitive component of the circadian clock. Science *311*, 1002–1005.

Yin, L., Wu, N., Curtin, J.C., Qatanani, M., Szwergold, N.R., Reid, R.A., Waitt, G.M., Parks, D.J., Pearce, K.H., Wisely, G.B., et al. (2007). Rev-erbα, a heme sensor that coordinates metabolic and circadian pathways. Science *318*, 1786–1789.

Yin, L., Joshi, S., Wu, N., Tong, X., and Lazar, M.A. (2010). E3 ligases Arf-bp1 and Pam mediate lithium-stimulated degradation of the circadian heme receptor Rev-erbα. Proc Natl Acad Sci U S A *107*, 11614–11619.

Zamir, I., Zhang, J., and Lazar, M.A. (1997). Stoichiometric and steric principles governing repression by nuclear hormone receptors. Genes Dev. *11*, 835–846.

Zhang, E.E., Liu, Y., Dentin, R., Pongsawakul, P.Y., Liu, A.C., Hirota, T., Nusinow, D.A., Sun, X., Landais, S., Kodama, Y., et al. (2010). Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. Nature Medicine *16*, 1152–1156.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., et al. (2008a). Model-based analysis of ChIP-Seq (MACS). Genome Biology *9*, R137.

Zhang, Y., Shin, H., Song, J.S., Lei, Y., and Liu, X.S. (2008b). Identifying positioned nucleosomes with epigenetic marks in human from ChIP-Seq. BMC Genomics *9*, 537.

Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C., and Bradley, A. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature *400*, 169–173.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, A., et al. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian Circadian Clock. Cell *105*, 683–694.

Zvonic, S., Ptitsyn, A.A., Conrad, S.A., Scott, L.K., Floyd, Z.E., Kilroy, G., Wu, X., Goh, B.C., Mynatt, R.L., and Gimble, J.M. (2006). Characterization of peripheral circadian clocks in adipose tissues. Diabetes *55*, 962–970.